ALGAL NUTRITIONAL REQUIREMENTS OF LARVAE OF *PAPHIA MALABARICA*

Thesis submitted to the
Cochin University of Science and Technology
in partial fulfilment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

UNDER THE FACULTY OF MARINE SCIENCES

BY

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(Register No. 2052)

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CENTRAL MARINE FISHERIES RESEARCH INSTITUTE

OCTOBER 2003

Dedicated to my

parents, sisters and Sumo

CERTIFICATE

This is to certify that this thesis entitled **'Algal nutritional requirements of larvae of *Paphia malabarica*'** is an authentic record of research work carried out by Gireesh R (Reg.No. 2052) under my guidance and supervision in Central Marine Fisheries Research Institute, in partial fulfilment of the requirements for the Ph D degree in Botany of the Cochin University of Science and Technology and no part of this has previously formed the basis for the award of any other degree in any University.

Date 23-10-03

DR. C.P. GOPINATHAN
(Supervising Guide)
Principal Scientist
CMFRI, Kochi.

DECLARATION

I hereby declare that the thesis entitled '**Algal nutritional requirements of larvae of *Paphia malabarica***' is an authentic record of research work carried out by me under the guidance and supervision of Dr. C. P. Gopinathan, Principal Scientist, Central Marine Fisheries Research Institute, in partial fulfilment of the requirements for the Ph D degree in Botany of the Cochin University of Science and Technology and no part thereof has been previously formed the basis for the award of any other degree in any University.

Date 23.10.2003

(**Gireesh. R**)

CONTENTS

Preface

Clams occupy an important position in molluscan resources, since they provide protein rich food much more than other bivalves, such as oysters and mussels. They are widely exploited from the natural beds all over the world, which may get depleted in due course of time. Clam culture is intensive in many parts of the world and hatchery production of seed was achieved in those countries, where it supported the commercial market.

In India, clams are widely exploited along the coastal belts. The clams *Villorita cyprinoids, Paphia malabarica, Meretrix meretrix, etc.,* are among the clam resources most widely exploited for their meat and shell, in the maritime states, especially in Kerala, Goa, Karnataka and Tamil Nadu. They form a livelihood for fishermen, irrespective of age and sex, as the effort for harvest is very simple. These clams are usually hand picked or collected by a small dredge or scoop net. Due to this, depletion of clams in natural bed occurred. The semi culture of clam seed in the estuary is valuable as it protects resources and hence the availability of seed is a prerequisite. The Central Marine Fisheries Research Institute has well developed larval rearing techniques for oysters and mussels. Seed production and ranching experiments of clams were conducted in Tuticorin Bay by the Tuticorin Research Centre of CMFRI to some extent.

In view of the export potential and natural depletion of the short neck clam, *Paphia malabarica*, experiments on seed production and ranching were conducted by CMFRI-MPEDA, in 1992. However, the optimum nutritional requirement for the larval rearing and spat production is not yet standardized. Hence, the present study made an attempt to standardize the algal nutritional requirements for the larval development and spat production of this clam. The micro algae which is the basic food in early larval stages of bivalves, hence evaluated for their performance on the basis of early settlement, survival and growth of larvae. The biochemical constituents of larvae during their development were also studied in detail. The effect of salinity and pH on algal nutrition for the larval development was also carried out to standardize the hatchery protocol. The details of the investigations are presented in **Five Chapters**.

The **Chapter I** give the General Introduction to the subject studied, where the details of resource potentials and review of earlier works discussed.

The **Chapter II** deals with larval rearing and spat production of *Paphia malabarica* using six micro algal species both as monodiet and combination of algal species in detail.

The **Chapter III** deals with filtration and clearance rate of larvae fed with different micro algal species.

The **Chapter IV** deals with the biochemical composition of both micro algae and larvae, in various stages of development.

The **Chapter V** deals with the effect of salinity and pH on algal nutrition for larval development and spat production.

The materials and methods and a brief introduction to the topic are included in respective chapters. The **Summary** and literature cited in the text is included in **Reference** section.

Acknowledgement

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Chapter 1

General Introduction

Molluscs in general, as a major fishery had always made an impact on economy from time immemorial all over the world. A wide variety of molluscs contribute edible and non-edible species, providing source of food, lime, pearl, decorative shells, raw material for chemical industry and constituent for medicinal preparations. It is estimated that in 2001 the world molluscan production was 29.58 million tonnes (FAO, 2001). Majority of molluscs inhabits marine biotopes and occurs from fresh water, brackish water, mangroves, inter tidal shelf and down to deep waters.

Molluscs form valuable fisheries in various parts of the Indian coast, providing shellfish as food and source of lime, pearls, and decorative shells and as components of drugs. Commercial cultivation of molluscs is one of the most and recent developments in aquaculture, which boosts economy of the nation. The annual production of molluscs is estimated as 3.9 lakh tonnes (CMFRI Ann. Rep., 1999-2000). The commercially important molluscs of India consist of oysters, mussels, clams and other bivalves, and gastropods, like the sacred chank (*Xancus pyrum*) and cephalopods. These resources are widely exploited by different methods such as hand picking, scoop net and standard dredging at different parts of the country. The Central Marine fisheries Research Institute has identified the importance of molluscan fishery and conducted several studies on identity, distribution, resource potential, biological

characteristics and culture methods of edible and pearl oysters, mussel and clam.

Among the exploited bivalve resources of India, clams are the most widely distributed and abundant species. In terms of production, they are foremost among bivalve resources. Several species belonging to five families (Veneridae, Arcidae, Donacidae, Solenidae, Corbiculidae) forms the clam resources and are being exploited along the east and west coast of India (Hornell, 1922; Alagarswami, 1966; Alagarswami and Narasimham, 1973; Naggapan and Nayar, 1974; Appukuttan *et al.*, 1985, 1988; Alagarswami and Meiyappan 1989; Rao *et al.*, 1989; Narasimham, 1991 a, b; Appukuttan, 1993, 1996). High nutritive value and their importance in earning of coastal fishing villages along with development of an export market, gave much attention to culture the clam resources during the last 15 years. In several estuaries on the west coast, this forms a major fishery. During the last five years, a very good demand has developed for the export of frozen clam meat. Various works have been done on aspects of biology, ecology, physiology and resources of important clam species in India. However, there are few studies only on nutrient requirements of bivalve larval stages, especially on clam larvae.

Among the venerid clams, widely exploited clam is *Meretrix meretrix*. Many workers had studied various aspects of this species such as biology (Rao, 1988), salinity tolerance (Rai, 1932; Ranade and Kulkarni, 1973; Ranade, 1964), effects of temperature and salinity on the oxygen consumption (Ranade, 1973), body component indices and

chemical composition (Nagabhushnam and Deshmukh, 1976), growth on transplantation (Rao and Rao, 1983), growth rate (Jayabal and Kalyani, 1986) and on larval rearing and spat production (Narasimham *et al*, 1988). *Meretrix casta* is the most extensively studied species related to biology (Abraham, 1953), spawning, growth on transplantation (Durve 1964, 1973) dimensional relationships, change of form and fatness (Durve and Dharmaraja 1965,1969,1972), age and growth (Balasubramanyam and Natarajan, 1988 b) and on biochemical composition (Gopalakrishnan *et al.*, 1977; Balasubramanyam and Natarajan, 1988 a). Same type of studies were also conducted in other clam species, such as *Katelysia opima* (Veneridae), *Villorita cyprinoids* (Corbiculidae), *Donax cuneatus* (Donacidae), razor clam *Solen kempi* (Solenidae) and blood clam *Anadara granosa* (Arcidae) by various workers. Studied on spawning and neuroendocrinology in *Paphia laterisulca* (Nagabhushnam and Dhamne, 1977a, b), maturity, spawning and age growth in *P. laterisulca* (Mane and Nagabhushnam, 1979), where as Winckworth (1931) made observations on the growth of *P. undulata*.

Kerala State stands first among all the maritime states in clam production with a catch of 28,600 tonnes of total clam landings in 1991. Major production centers are Vembanad and Ashtamudi Lakes. In Ashtamudi estuary, the venerid clam, *Paphia malabarica* forms the major exploited fishery (1723 in 1991- 31,000 tonnes in 2001). Surveys conducted by many agencies in these Lakes have yielded valuable informations on clam fisheries.

The taxonomic position of the short neck clam, *Paphia malabarica* Chemnitz, utilized in the present study, is given below.

Class : Pelecypoda

Order : Eulamellibranchiata

Sub-order : Heterodonata

Series : Veneracea

Family : Veneridae

Genus : *Paphia*

Species : *malabarica*

Paphia malabarica, called as " Poovan Kakka" in Malayalam is widely exploited in Ashtamudi Lake. The shell is more or less elongate, smooth or concentrically sculptured with narrow elongated hinge. Hinge area is short with narrowing teeth. Pallial sinus is moderately deep and ' U ' shaped. The shell attains a size of 20 mm on maturity. This clam has spawning season from September to January. It is known that species of *Paphia* have more life span than other clams.

A good fishery of *P. malabarica* is seen in Karwar, North Kanara river mouths and also in Vembanad - Ashtamudi Lake in Kerala (Kripa and Mathew, 1993). It occurs in depth of up to 4 m in sandy mud. During low tide, fishermen collect these clams from beds using scoop nets in one hand against the current and clams are pushed into the net with the other hand.

In *Paphia malabarica*, Rao (1988) studied on maturity, spawning, age, growth and dimensional relationships, on fishery (Appukuttan, 1996), ecology (Parulekar, 1984), calorific value (Vijaya Raghavan *et al.,* 1975) salinity tolerance (Ram, 1998) and biochemical composition (Appukuttan and Aravindan, 1985.)

Paphia malabarica is exploited in considerable quantities along the west coast of India. Appukuttan *et al.,* (1996 and 1999) had dealt with the clam resources of Ashtamudi Lake and reported on shortneck clam, *P. malabarica* as an emerging resource of commercial importance. They are usually used for clam meat and shell in cement industry. This species is also suitable for integrated farming. Their contribution remains 80-90% of frozen clam meat export from India. Overexploitation and the seed availability in nature is a problem. Although, attempt on larval rearing and spat production and sea ranching of *P. malabarica* was done (Narasimham, 1993), the basic hatchery technology for seed production is not yet developed. During the present study, larval rearing was tried with unialgal food *Isochrysis galbana* on experimental basis as the optimum density of feed suitable for, growth during each of its larval stages i.e., *'D'* shape, umbo, veliger, pediveliger and spat. In the present study evaluation of different micro algae in terms of physiological and biochemical characters is carried out, during different stages of larval cycle.

Micro algae are considered as the primary food of marine invertebrates, it supplies both energy and essential nutrients in the form of protein, carbohydrates and lipids (Whyte, 1987). Environmental conditions under which micro algae grow greatly affect the biochemical compositions and may alter the energetic and nutritional value of the organisms. The food value of micro algae depends upon the consumability of the rearing larvae. Micro algae species, such as *Isochrysis galbana, Pavlova lutheri, Tetraselmis gracilis, Platymonas* (*Tetraselmis*) *spp* and *Dunaliella spp.* induced more rapid growth of both clam and oyster larvae in hatcheries. *Nannochloropsis spp* was recognized as essential component of live feed required in the rearing operation of many bivalves (Mourente *et al.*, 1990; Seto *et al.,* 1992 and Nelson *et al.*, 1992). In Japan, *Nannochloropsis spp* is cultured in large scale in outdoor tanks year round to feed rotifers and to establish a good source of protein for the production of fish larvae and bivalves (Morizane, 1991).

Research on mass culture of micro algae has been carried out in many parts of the world for the past fifty years. For many years, plant physiologists, algologists and bio-engineers have had a special interest in the possibilities of mass culturing micro algae. In the past, main focus has been on single cell protein (SCP) production, but in recent years, many other potential application for large scale culture have been advanced including waste water treatment, the production of extractable commercial chemicals, closed life support systems, bioconversion of solar energy and in aquaculture.

In recent years, there has been renewed interest in producing single cell protein by mass culturing the unicellular micro algae such as diatoms and nannoplankton flagellates for feeding the larvae of crustaceans, molluscs and fishes. As is well known the success of any hatchery operation depends mainly on providing the required species of micro algae and zooplankton suitable for the larvae. The larvae of prawns and fishes prefer diatoms as the basic food while the molluscs feed on the nannoplankton flagellates, measuring less than 10μ during its larval stages.

Even after two decades of research on the formulation of micro diets to replace live food in larviculture, there is only a limited success (Watanabe and Ackman, 1983). Presently micro algae culture provide the only practical method of mass feeding filter feeders, and are assumed to be natural food and have been used successfully in shellfish culture (Loosanoff and Davis, 1963). Micro algae vary in their proportions of protein (10-60%), carbohydrates (5-27%) and lipid (7-23%). They are used for larval nutrition usually in the nannoplankton size range (2-20μ). Many of them exhibit a large variability in composition, which is dependent on species and culture conditions (Enright *et al.*, 1986; Mortensen *et al.*, 1988; Sukenik *et al.*, 1989; Emdadi and Berland, 1989). Important members of micro algae commonly used in hatcheries for larval rearing are: diatoms (Bacillariophyceae) such as *Skeletonema costatum* and *Chaetoceros spp.* and phytoflagellates such as *Isochrysis galbana, Chromulina spp.* and *Pavlova spp.* (Haptophyceae) and *Dicrateria*

(Chrysophyceae) and green phytoplankters such as *Nannochloropsis spp.* and *Chlorella spp.*. The usage of micro algae as live feed depends mainly on the nutritional quality as well as their tolerance to temperature, salinity and light conditions especially while maintaining as stock culture, indoor and outdoor mass culture systems.

Literature on larval feeding studies, primarily conducted in temperate regions, reported that species of *Isochrysis, Monochrysis, Pavlova* and *Platymonas* in unialgal culture and in mixtures are suitable feed for molluscan larvae (Davis and Guillard, 1958; Walne, 1963,1966,1974; Lossanoff and Davis, 1963; Albentosa, 1993, 1996). Diatom bloom in the natural seawater tends to satisfy the feed requirements of the juvenile bivalves (Hidu and Richmond, 1972) but is generally regarded as a weed species of low food value. This is due to the inability of the majority of the larvae to digest the siliceous cell wall. But these diatoms can be used as a food for late larvae of all kinds of bivalves. Sometimes mortality may occur due to the large size of the cells and difficulties encountered in their ingestion or due to some other deleterious factor in the culture. Food values of certain species of algae have been conflicting in some cases as a result of variations in the culture conditions such as light and temperature especially in winter seasons.

In tropical regions, in most of the studies, the larval growth rate, percentage of survival, spat production and setting success judged the food value of phytoplankton. It was suggested that various micro algae are not equal in nutritive value and that an algal diet composed of two or

three species produce a better food source than any individual species. In one such study, Chu *et al.*, (1982) examined that a mixture of *Chlorella, Pyrominionas* and *Pseudoisochrysis* can optimize growth and reduce the setting time of oyster larvae to 8 – 10 days. While traditional algal diet of *Isochrysis galbana* and *Pavlova lutheri* results in setting of larvae in 13-15 days (Guillard, 1958). Availability of live feed, which is the basic food for all bivalve larvae, is thus still remains as a problem. Alternate feeds, encapsulated, enriched, dried algal powder forms were tried in other species. But it is too costly and yield is not encouraging to perform as a hatchery technology. Hence, micro algae remain as most suitable feed in bivalve culture till date.

Several attempts have been made to correlate the biochemical contents of phytoplankton to suitability as food for herbivores (Parsons *et al.*, 1961; Walne, 1970; Epifanio, 1979). Even though chemical compositions of the algal species were qualitatively similar, there were some difference in the quantity of some amino acids, proteins and total fatty acids. So it is necessary to identify the suitable feed, which have optimal requirements and that which suits to each larval stage of bivalves. Hence the present study is an attempt to correlate the minimum feed requirement and their effect on biochemical composition in larval stages of the clam, *Paphia malabarica.* Each species varies according to their nutrient requirements as well as environmental characters. The larval rearing in an appropriate salinity is another major constraint in hatchery production. Hence an attempt has been made on the effect of salinity and pH during larval rearing of this clam.

Chapter 2

2. Larval rearing and Spat production of *Paphia malabarica* (Chemnitz)

2. 1. Introduction

Among the exploited molluscan resources, oysters, mussels and clams were by far the most important and abundant. They form a valuable fishery world wide to support the commercial markets. Molluscs especially bivalves provide meat as food and source of pearl, lime, cement and decorative shell and also as a component of medicinal drugs. The rearing of larvae and juveniles of bivalves has been tried and standardized in hatcheries worldwide. Many workers in bivalve culture, especially in oysters and mussels, carried out successful conditioning and rearing methods. Recently, the same methods have also been tried on culture clam species of aquaculture importance. Loosanoff and Davis (1950) have worked on twenty species of molluscs. The optimal requirement of commercially important bivalve species is still not fully studied and is an area of major constraint. As the species vary geographically and due to environmental conditions, it is difficult to adapt a standardized method for larval rearing and spat production. Hence, an attempt has been made to investigate the rearing technique of *Paphia malabarica* a species of commercial importance in India.

Paphia malabarica is the major clam species, which enters the commercial market as frozen clam meat for the past few years. Although an attempt on larval rearing of *Paphia malabarica* and seed ranching has been

worked out on an experimental basis (Narasimham, 1991), no other information is available about optimal requirements of feed and spat production of this particular species. Since the biological, physiological and biochemical variations are species specific, the metamorphosis during larval life will give a growth pattern of individual species. This will be beneficial to understand the optimal food requirements for the culture of this species.

2. 2. Material and methods

2. 2. 1. Brood stock maintenance and larval production

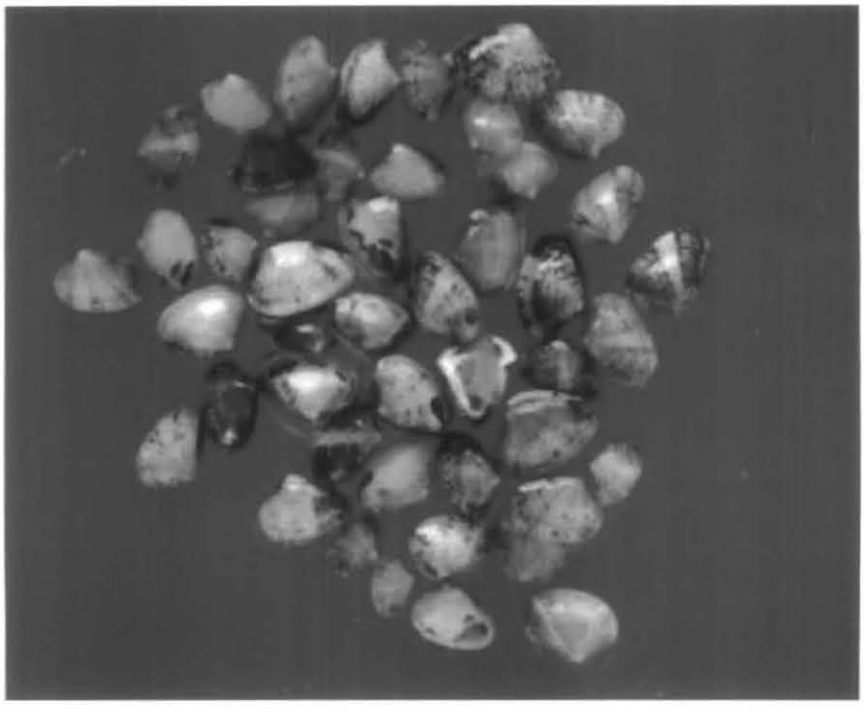
Brood stocks of clams (Plate 1) were collected by using scoop net, from Dhalavapuram in Ashtamudi Lake, south west coast of India (Latitude 8°00 N and Longitude 69° E) (Salinity 29 ± 2 $^{o}/_{oo}$, Temperature 28 ± 2° C, pH 7.9 –8.1) and transported to the hatchery at Tuticorin Research Centre of CMFR Institute (Latitude 8° 00 and Longitude 67°00) during the period May-November 2001. The clams were acclimatized for two days and monitored during this time for occurrence of natural spawning. If not spawn, 50 % were transferred to conditioning room where temperature was kept at 20°C and mixed diet of *Isochrysis galbana* and *Chaetoceros calcitrans* were given twice a day. Other 50 % of the clams were kept in 500-litre Perplex tank in hatchery to observe, if there are any chance of spawning in natural condition the next day. Otherwise, brood stock was continuously fed with mixed algal culture consists of *Isochrysis galbana* and *Chaetoceros calcitrans* for one

Plate 1

Paphia malabarica **(Chemnitz)**

Adult clam: 20 mm size.

Plate 1



week before attempting thermal inducement. Seawater was changed daily and salinity was kept under ambient condition of about 32 – 35ppt.

About 50 clams were selected from the conditioning room for induced spawning, in each experiment. The seawater used was thoroughly filtered using 40 μ sieves. The water temperature was raised to 4 –5 ° C above normal temperature. After 45 minutes to one hour, the whole clams were transferred to a tank, containing filtered seawater and temperature and salinity kept normal (33 $^{o}/_{oo}$ and 27 –29 ° C).

The clams were allowed to spawn for 30-45 minutes in each experiment. Later, whole clams were transferred to another tank. Both sperms and eggs were allowed to fertilize for few minutes. The eggs were transferred through a sieve (60 μ) to remove unfertilized eggs and debris. The fertilized eggs were washed using fresh filtered seawater twice and transferred to 10-litre flask. The number of fertilized eggs was counted using a coulter counting chamber and the density of larvae were determined. Usually 2 to 3 million larvae were observed in each spawning during the experiments. The healthy larvae were transferred to 1 tonne FRP tank for rearing and observed till it reached '*D*' shape'. The tank was covered with black cloth to prevent any algal growth and well aerated. About 95-97% of the larvae attained *D*-shape and larval rearing experiments were conducted from this stage onwards (Plate 2).

Plate 2

a) Fertilized egg

b) 2 celled stage

c) 3 celled stage

d) 4 celled stage

e) *D* shape larvae

f) Umbo

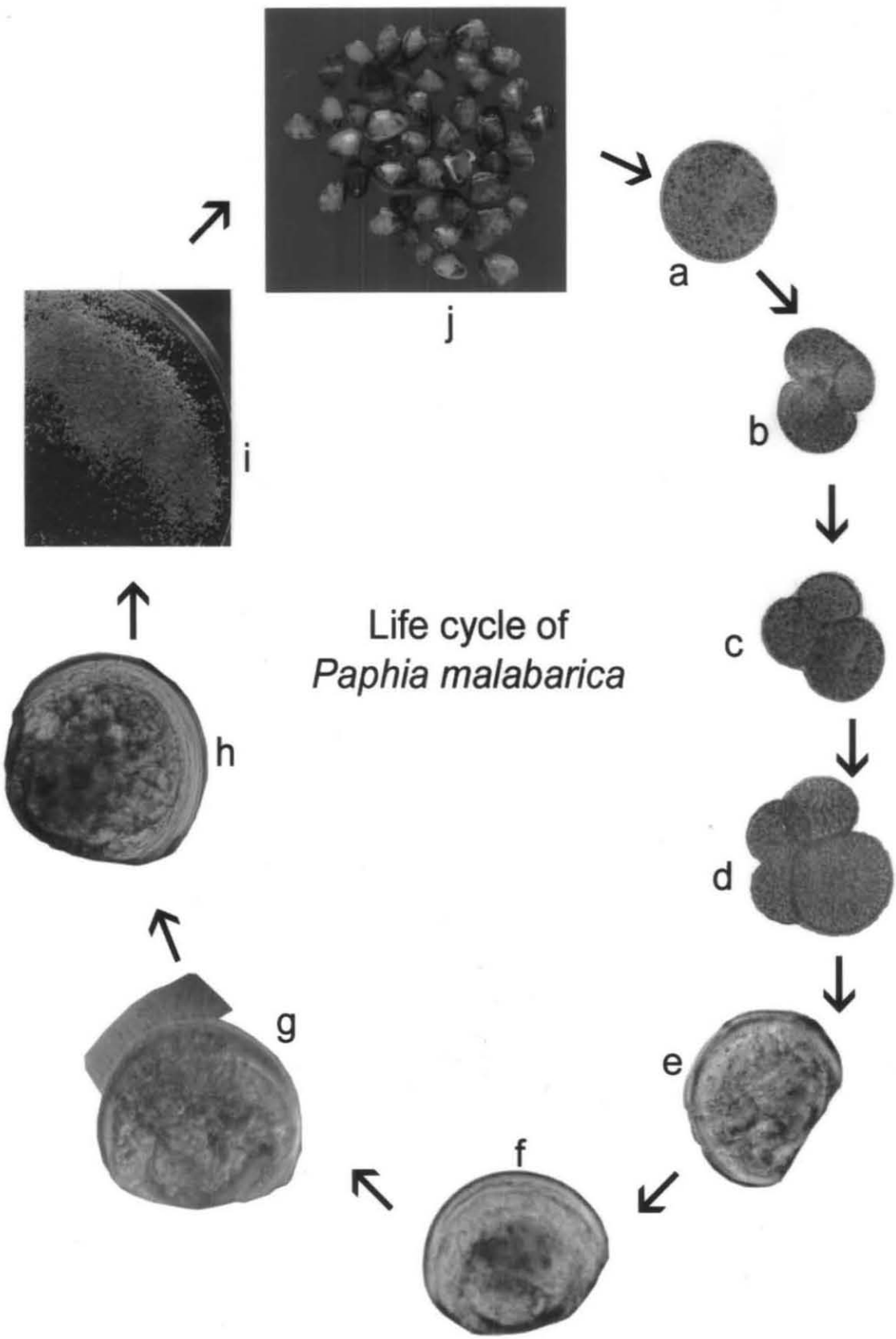
g) Pediveliger

h) Settled spat

i) Spat- one month old.

j) Adult clam

Plate 2



2. 2. 2. Larval rearing with mono algal diets

Experiment I

About 10,000 '*D*' shaped larvae were transferred to each 10-litre capacity glass jar for rearing using the mono algal diet, *Isochrysis galbana* at 3000, 4000, 5000 and 6000 cells ml^{-1} concentrations. The salinity and temperature were kept at ambient levels, 32-33 $^{o}/_{oo}$ and 29-31 °C respectively. This micro algal species has been considered as a diet by many workers and used in almost all molluscan hatcheries. Hence, *Isochrysis galbana* was taken as control diet for evaluation of different diets in the following experiments.

Experiment II

This experiment was conducted based on the results obtained in experiment I. The density of larvae in each trough was kept at 1 larva/ml. The volume of filtered seawater was 10 litres in 25 litre capacity troughs. '*D*' shaped larvae were transferred to each trough and fed with different mono micro algal diets. The micro algal species used as feed in this experiment were *Isochrysis galbana, Chaetoceros calcitrans, Tetraselmis gracilis, Nannochloropsis salina, Dunaliella salina* and *Dicrateria inornata* (Plate 3). The micro algal species were maintained both as stock culture using Conway or Walne's medium and indoor mass culture for the larval rearing (Plate 4). Initial measurement (length and breadth) of the larvae was taken using micrometer. However, the anterior posterior measurement is only taken for

Plate 3

Micro algal species

a) ***Chaetoceros calcitrans***

b) ***Dicrateria inornata***

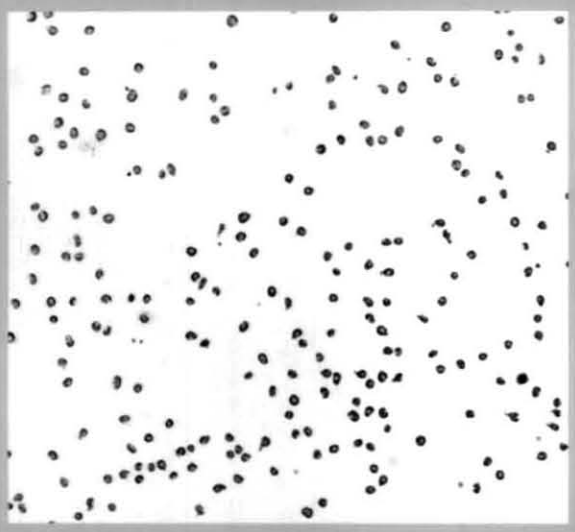
c) ***Tetraselmis gracilis***

d) ***Dunaliella salina***

e) ***Nanochloropsis salina***

f) ***Isochrysis galbana***

Plate 3



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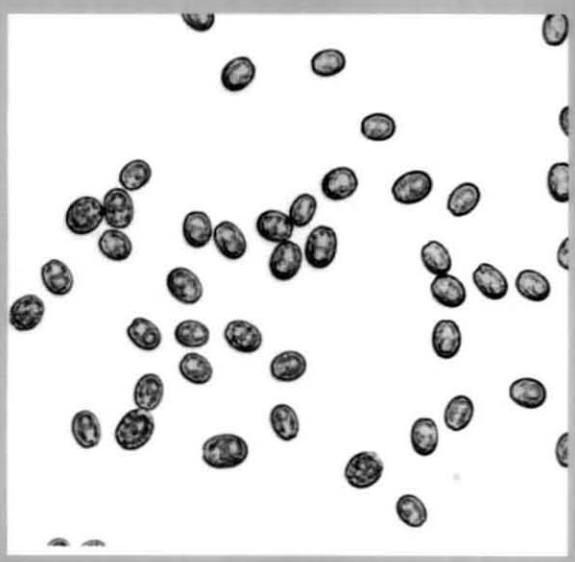
b



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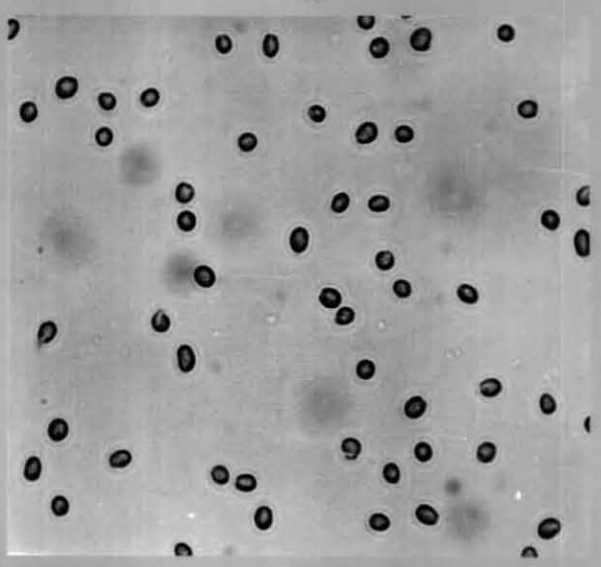

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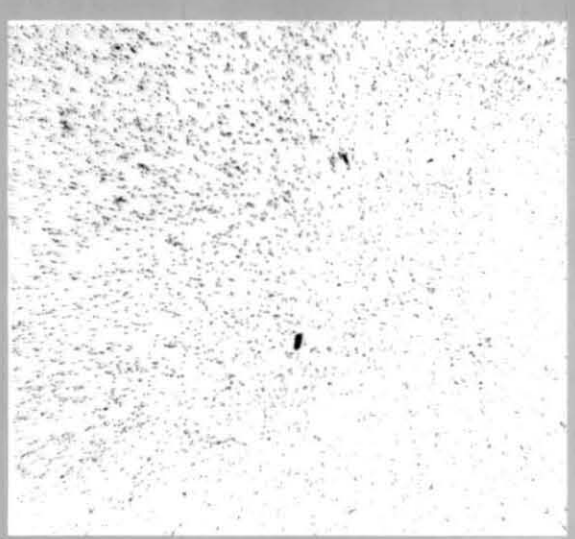

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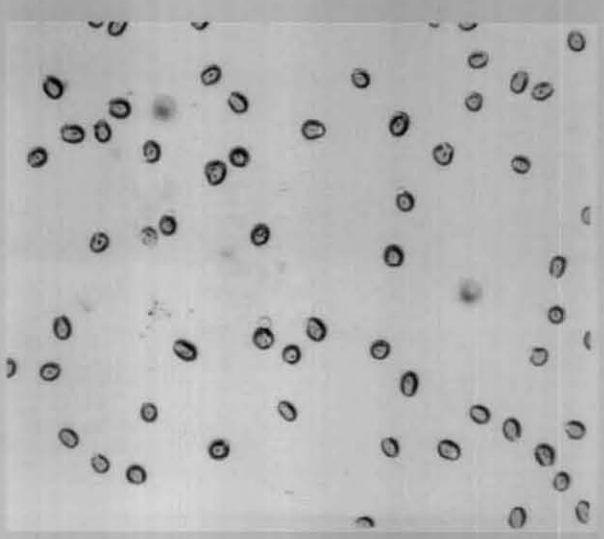


f

Plate 4

Micro algal culture

a) Stock culture of micro algae

b) Indoor mass culture of *Isochrysis galbana*

c) Indoor mass culture of *Tetraselmis gracilis* and *Nannochloropsis salina*

Plate 4

a

b

c

test of significance between the diets. The feeds were given in equal cell concentrations (10,000 cells ml^{-1}). The experiments were completed after the settlement of larvae in each feed regime. Number of days for settling of the larvae in each algal species and length-breadth measurement in each stage of life cycle of larvae such as *D*-shape, umbo, veliger and spat were noted (Plate 1).

2. 2. 3. Larval rearing with combination of two algal species

Experiment III

This experiment was conducted to evaluate combinations of two algal species during larval rearing. The procedure of experimental set up is same as described in the experiment II. The density of larvae in each trough was kept at 1 larva/ml. The volume of filtered seawater was 10 litres in 25 litre capacity troughs. *D*-shaped larvae were transferred to each trough and fed with combination of two micro algal species in equal ratio (1:1). The micro algal species used as feed in this experiment were *Isochrysis galbana*, *Chaetoceros calcitrans*, *Tetraselmis gracilis*, *Nannochloropsis salina*, *Dunaliella salina* and *Dicrateria inornata*. Initial measurement (length and breadth) of the larvae was taken using micrometer. However, here also, the anterior posterior measurement is only taken for test of significance between the combinations of diets. The feeds were given in equal cell concentrations (i.e., 10,000 cells/ml). The experiments were completed after the settlement of larvae as spat in each feed regime. Number of days for settling of larvae in

each algal combination and length-breadth measurement in each stage of life cycle of larvae such as *D*-shape, umbo, veliger and spat were noted.

Experiment IV

In this experiment, based on the results obtained in the above experiments, the algae, which promoted high survival rate, growth and growth rate, were used as diets to evaluate the optimum cell density for larval rearing. In this experiment larvae were fed with *Nannochloropsis salina*, *Isochrysis galbana* and a combination of these two species (ratio 1:1). Ambient temperature (32 ± 2°C), pH (8.0-8.2) and salinity (29 ± 2 $^{o}/_{oo}$) were maintained as normal condition. The concentration of feed given in this experiment was same as in the experiment I. Initial length and breadth measurement of *D*-shape larvae were noted. Seawater was changed daily prior to feeding micro algal species. The initial and final concentration of feed in the experiment trough were counted daily using a haemocytometer, to determine the consumability of micro algal species. The same method was adopted in all the above experiments.

The other environmental parameters, pH, nutrients, dissolved oxygen, and chlorophyll *a* in the seawater used were estimated during these experiments. Whole experiments were statistically tested for significance in each diet both single and in combination of two species.

2. 2. 4. Evaluation of micro algae for spat rearing

The settled spat in the mean size 1.00 ± .20 mm were reared for one month with monoalgal diets and combination of two species as mentioned in the larval rearing experiments above.

The observed mean temperature, salinity and pH were 28 ± 3°C, 30 ± 3 $^{o}/_{oo}$ and 8.0 – 8.3 respectively. The other environmental parameters pH, nutrients, dissolved oxygen, and chlorophyll *a* in the seawater used was studied during this experiment. The final anterior posterior measurements and weight of spat is taken on day 30 to calculate growth and growth rate pattern, to evaluate the efficiency of different micro algal diets. The algal species used in the present study was same as represented in the larval rearing experiments above.

2. 3. Results

2. 3. 1. Larval rearing with mono algal diets

Experiment I

Growth

The observed mean length of settled spat, after initial settlement on 13th day of experiment and on final settlement on 15th day is represented in Table 2.1. Although growth produced by 3000 and 5000 cells/ml concentrations were equal and more than half of produced by 4000 cell/ml, the survival rate was comparatively less. One-way ANOVA demonstrate that the growth of larvae was significantly influenced by algal cell concentrations.

Average size of umbo produced when fed with 4 x 10^3 cells ml^{-1} concentrations was of 112 X 102 µm, where as 116 X 102 µm in 3000-cells/ml concentrations, 127 X 120.5 µm in 5000-cells/ml concentrations and 112 X 103 µm in 6000-cells/ml concentrations.

There was a rapid growth from umbo stage onwards in case of 4 x 10^3 cells ml^{-1} concentrations and settled after 7 days. On 13^{th} day and average size of settled spat was 192 X 175 µm. The average size measured in other concentrations of feed was 176 X 163 µm (5 x 10^3 cells ml^{-1}), 176 X 150 µm (3 x 10^3 cells ml^{-1}) and 166 X 150 µm (6 x 10^3 cells ml^{-1}). The average size of spat were measured as 643.2 ± 130 µm, 693.6 ± 124 µm and 577.6 ± 42 µm in case of 3000, 5000 and 6000 cells/ml concentrations respectively on day 18 on the completion of this experiment.

The size frequency percentage pattern (Fig. 2. I) shows more spat were settled in the maximum range of 180-190 µm in the cell density 4 x 10^3. The observed frequency was 94 %, 15 %, 12 % and 2 % in the densities 4, 5, 3 and 2 x 10^3 cells/ml. However, size frequency in the range 170-180 µm shows maximum number of spat in cell densities 3 and 5 x 10^3 cells/ml with 88 and 85 % respectively. Low size group in the range 160-170 µm was observed in cell density 6 x 10^3/ml (89%).

Growth rate

The maximum growth rate was observed in the cell density 5 x 10^3 in the early developmental stage. It is 12.1 µm/day in the above density of *Isochrysis galbana*. The growth rate from umbo to spat thereafter shows a slow development while it is observed a high growth rate in the same stage in 4 x 10^3. The observed growth rate in other densities was summarized in Table 2. 2. The growth rate on statistical analysis shows significance between the treatments ($P \geq 0.05$). Table 2. 3.

Survival

The larvae reared at cell density 4 x 10^3 cells/ml produced a survival rate of 32.4% in the present experiment. The other concentrations such as 3000, 5000 and 6000 cells/ml produced a survival rate of 18.1%, 13.1% and 0.68% respectively. (Fig. 2. 2). The maximum survival rate of umbo (78 %) till settlement was observed in cell density 5 x 10^3 cells/ml (Fig. 2. 3). Even a high survival of 88% umbo in 3 x 10^3 cell density, the overall spat settled was low compared to above cell density.

Table 2. 1. Mean size of clam larvae reared at different densities of *Isochrysis galbana*

Days ↓	***Isochrysis galbana* at different concentrations (x 10^3 / ml)**			
	Mean Size (µm) (anterior posterior measurement)			
	3 x 10^3	**4 x 10^3**	**6 x 10^3**	**5 x 10^3 (control)**
2	88 ± 8.2	83 ± 5.2	83 ± 5.2	86 ± 2.3
3	116 ± 10.1	112 ± 8.1	115 ± 8.0	127 ± 2.0
5	122 ± 5.0	114 ± 4.5	122 ± 4.5	135 ± 3.0
7	130 ± 2.0	128 ± 2.0	130 ± 2.5	171 ± 2.0
11	162 ± 2.5	147 ± 2.0	150 ± 2.0	182 ± 2.5
15	176 ± 1.5	187 ± 2.0	166 ± 4.0	184 ± 2.0

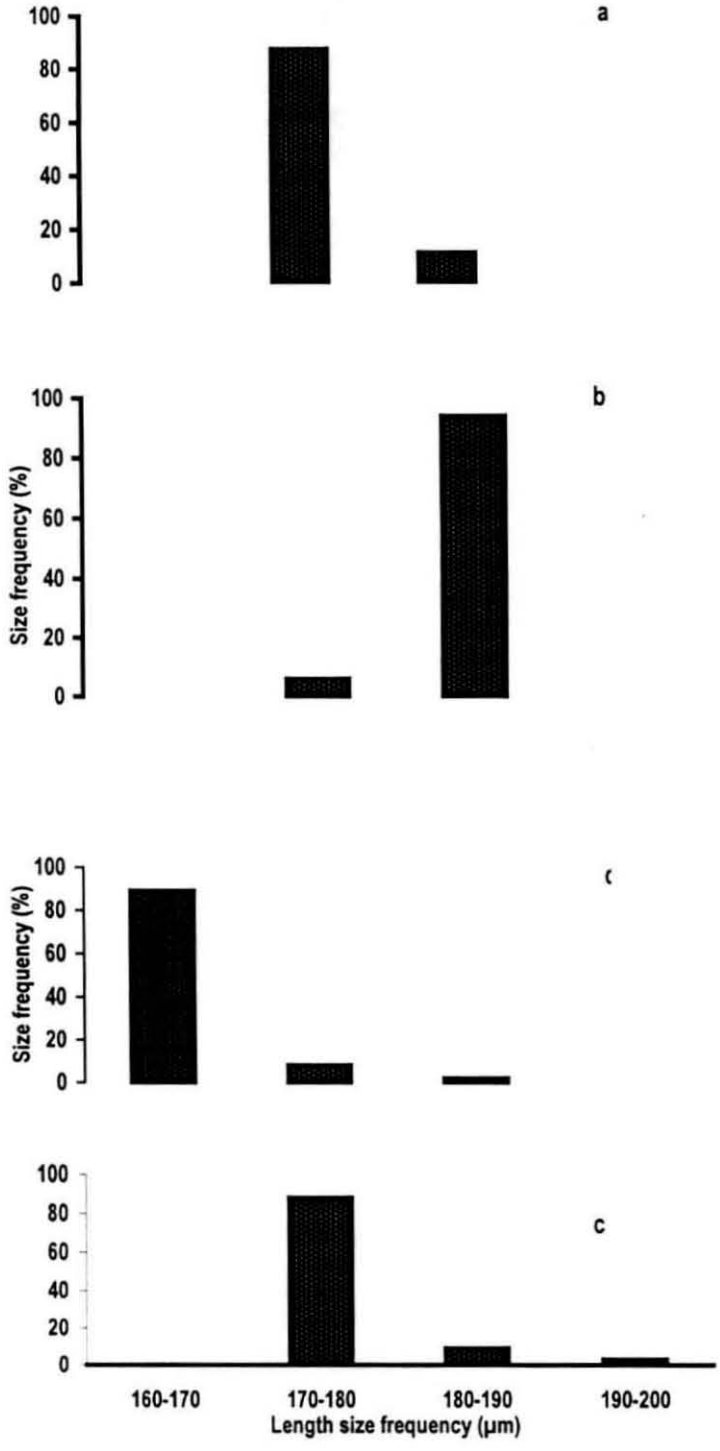
Table 2. 2. Growth rate of clam larvae fed with *Isochrysis galbana* at different cell densities

Larval stage ↓	Growth rate (µm)/day			
	3 x 10^3	4 x 10^3	5 x 10^3	6 x 10^3
D - umbo	6.0 ± .1	6.4 ± .2	12 ± .1	6.7 ± .2
Umbo – spat	5.8 ± .5	7.3 ± .3	1.6 ± .2	4.3 ± .3
D - spat	5.9 ± .3	6.9 ± .2	6.5 ± .1	5.5 ± .4

Table 2. 3. ANOVA for growth of larvae when fed *Isochrysis galbana* at different concentrations

Stage	Source of variation Main effects	Sum of squares	Df	Mean sq	F Ratio	Significance level
Larvae	Day	2.53874	6	5077471	2465	0.000
	Concentration	376930.0	5	125643.364	61.02	0.000

Fig. 2. 1. Mean size frequency distribution of larvae reared in different densities of *I. galbana*



a. 3 x 10^3, b. 4 x 10^3, c. 6 x $10^3$and d. 5 x 10^3 cells/ml.

Fig. 2. 2. Survival rate of umbo during development and spat settled in different densities of *Isochrysis galbana*

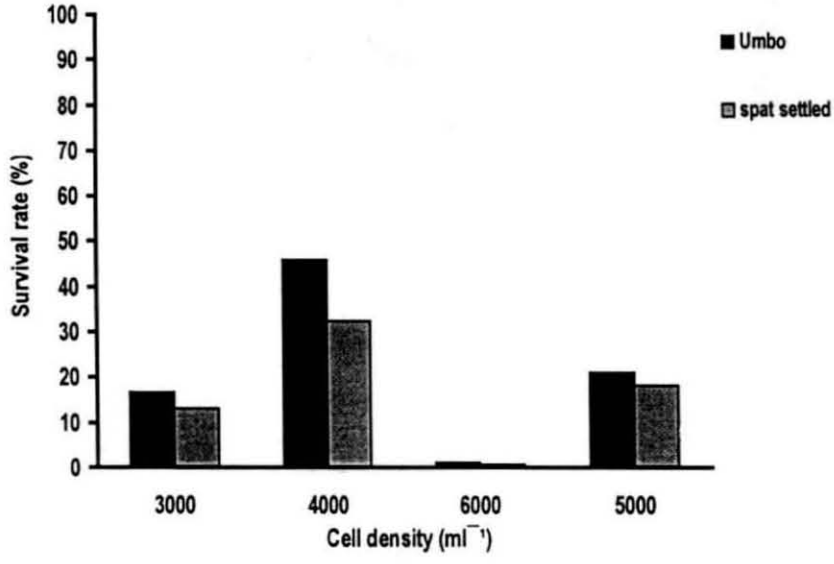
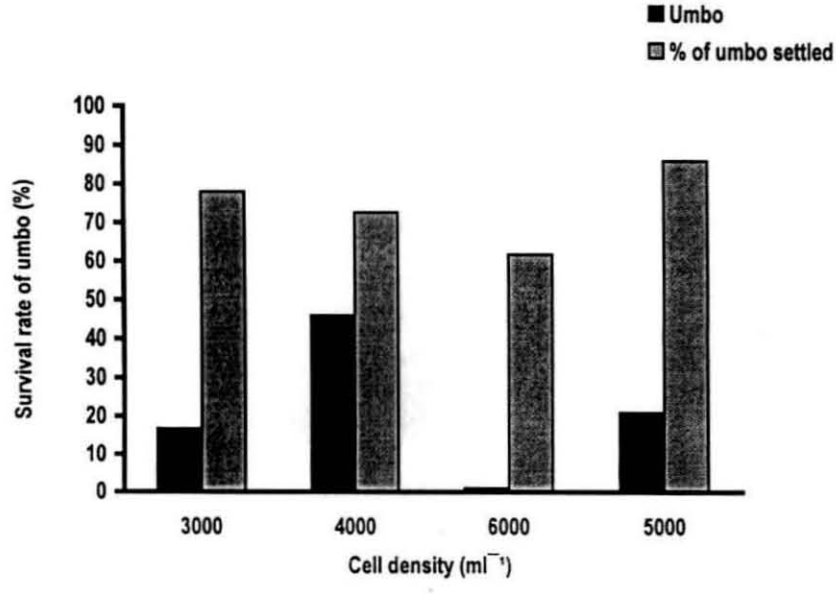


Fig. 2. 3. Percentage of umbo settled, when reared with *Isochrysis galbana* at different densities



Experiment II

Larvae fed with *Nannochloropsis salina*

Growth

The larvae fed with *Nannochloropsis salina* shows maximum growth at 5000-cells/ml concentrations. The observed mean sizes were in early umbo stage (147.8 x 122 µm), pediveliger (222.2 x 180 µm) and spat (342 x 298 µm) (Fig. 2. 4). Average minimum size of first settled spat measured as 302 x 287 µm and maximum of 382 x 308 µm on 9^{th} day. Size of spat on 17^{th} day was measured as 656.8 ± 4.5 x 624.8 ± 2.6 µm.

Fig. 2. 4. Mean size of larvae fed with *Nannochloropsis salina*

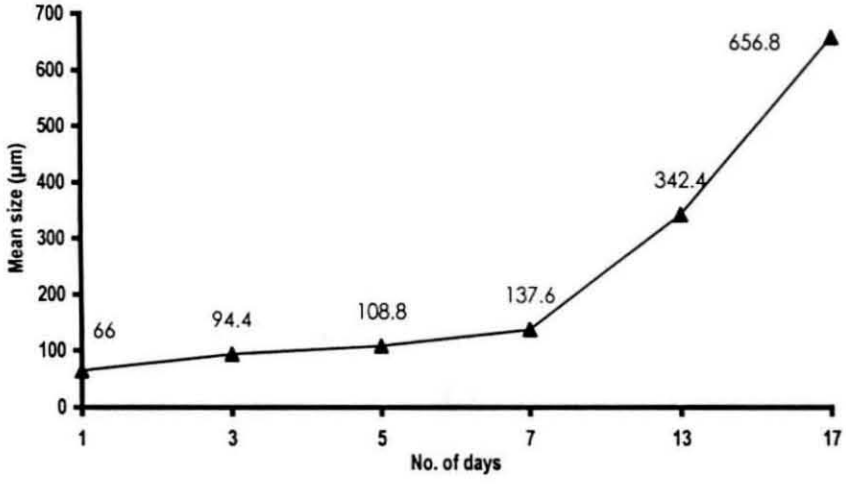


Table. 2. 4. Mean growth rate and survival rate of spat

Stage	Mean growth rate (µm/day)
D – umbo	8.8 ± 0.21
umbo-spat	38.2 ± 0.11
D - spat	19.8 ± 0.21
Settling started (day)	9
Settling completed (day)	11
Rate of spat production (%)	32

Growth rate

Growth rate was measured as 8.8 µm/day during development from *D* larvae to umbo and 38.2 µm/day from umbo to spat. Overall growth rate during the larval development till settlement was 19.8 µm/day (Table 2. 4).

Survival rate

High survival rates were noticed when larvae fed with *Nannochloropsis salina*. About 48 % of *D*-shape larvae metamorphosed into umbo. No other algal species produced such a high survival rate during this study period. About 66 % of umbo settled with an initial settlement on 9^{th} day after spawning and 32 % of total larvae settled after their planktonic life (Fig. 2. 5 and 2. 6).

No. of days for settlement.

Larvae reared using this species recorded the first settlement on 9^{th} day (63 %) and rest of larvae settled on 10^{th} day.

Fig. 2. 5. Percentage survival of umbo and spat, when fed with mono algal diets

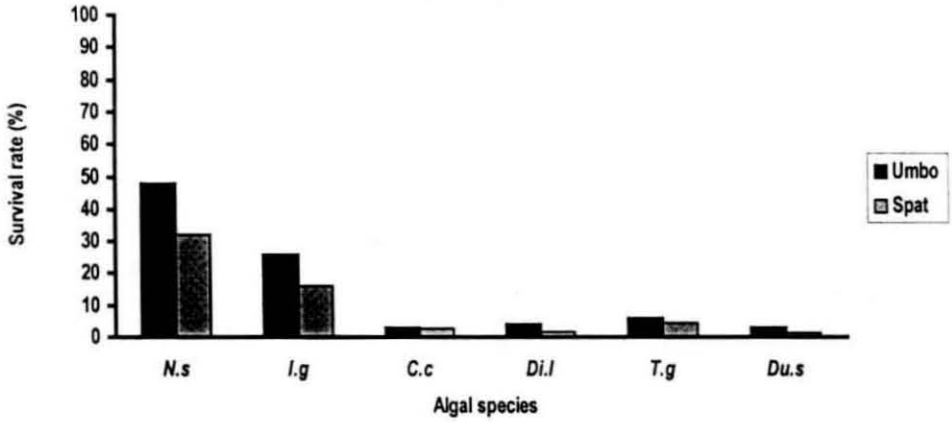
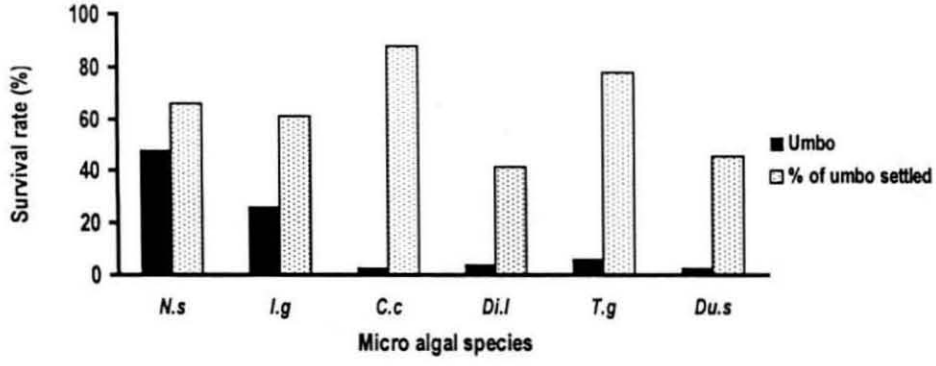


Fig. 2. 6. Percentage of Survival of umbo and % of umbo settled, when fed with mono algal diets



Larvae fed with *Dicrateria inornata*

Growth

Larvae showed an increase in size when fed on this species with a mean size of umbo 124 x 104.4 µm and pediveliger 128 x 112 µm. Here the size of umbo and pediveliger were small compared to it in other micro algal feeds during corresponding days. The observed mean size of spat settled on day 12 was 148 µm (Fig 2. 7). The observed minimum and maximum mean size of spat settled on day 9 was 146 µm and 156 µm respectively.

Fig. 2. 7. Mean size of larvae when fed *Dicrateria inornata*

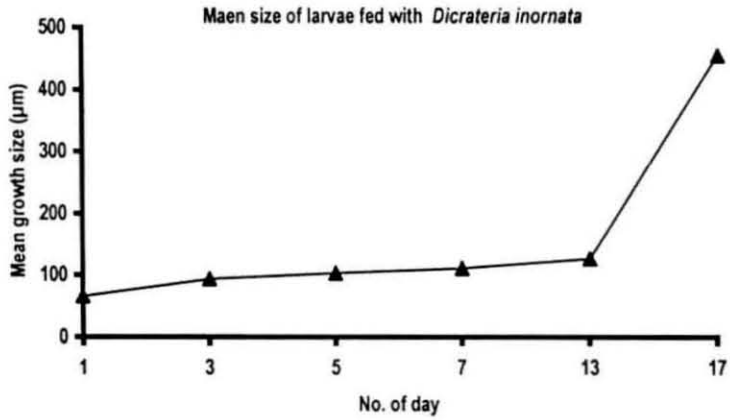


Table 2. 5. Mean growth rate and survival rate of spat

Stage	Mean growth rate (µm/day)
D – umbo	8.6 ± 0.2
umbo-spat	2.3 ± 0.2
D - spat	6.0 ± 0.2
Settling started (day)	12
Settling completed (day)	13
Rate of spat production (%)	1.7

The observed minimum mean size was 132 x 120 µm and maximum mean size of the spat settled was of 160 x 146.4 µm on day 15th of settlement. This algal species shows a deprived performance in larval rearing of *P. malabarica*.

Fig. 2. 8. Mean size of larvae fed *Chaetoceros calcitrans*

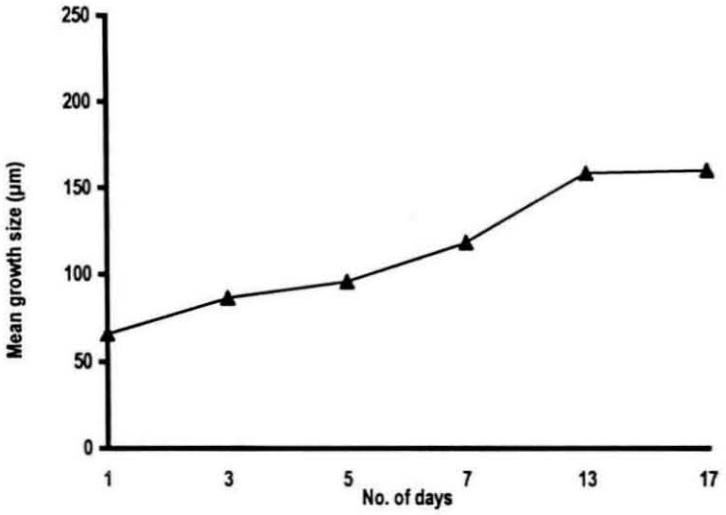


Table 2. 6. Mean growth rate and survival rate of spat

Stage	Mean growth rate (µm/day)
D – umbo	8.6 ± 0.4
umbo-spat	2.3 ± 0.5
D - spat	4.9 ± 0.2
Settling started (day)	14
Settling completed (day)	16
Rate of spat production (%)	2.6

Growth rate

Low growth rate was observed in this species when fed with larvae. The observed growth rate in early stage, *D*-shape to umbo was measured with a rate of 8.6 µm/day. Thereafter, the growth rate (2.3 µm/day) decreased very much till settlement, with a final mean growth rate of 4.9 µm/day (Table 2.6).

Survival rate

About 3 % larvae were alive during umbo stage. There after, the larvae gained stability in development with 88 % of spat settlement. The total survival from umbo to spat, when fed *Chaetoceros calcitrans,* was a poor 2.64% (Fig. 2. 5).

No. of days taken for settlement

The whole spat settlement occurred on 14^{th} day as larval density was poor from umbo stage.

Larvae fed with *Tetraselmis gracilis*

Growth

In initial stage of rearing, when fed with this species, showed a moderate growth in umbo stage. The observed umbo mean size was measured 124 x 124 µm., which was same as in larvae fed with *Dicrateria*

inornata and *Dunaliella salina*. However, observed mean size of settled spat was lower than the above species with a mean size of 300 x 272 µm. Here the viable larvae showed different size groups during their development and hence indirectly influenced spat settlement. Fig 2. 9 represents mean growth size of various stages of larvae till settlement.

Fig. 2. 9. Mean size of larvae when fed *Tetraselmis gracilis*

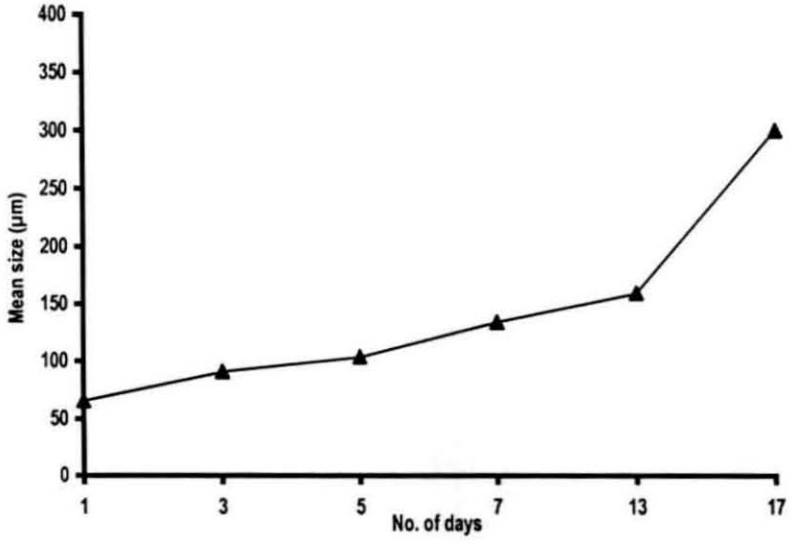


Table 2. 7 Mean growth rate and survival rate of spat

Stage	Mean growth rate (µm/day)
D – umbo	5.0 ± 0.01
umbo-spat	5.0 ± 0.5
D - spat	5.0 ± 0.2
Settling started (day)	13
Settling completed (day)	15
Rate of spat production (%)	4.5

Growth rate

This algal species produced a high growth rate initially and there after a decline in growth. Different size groups of larvae after the umbo stage was due to viability of larvae on feeding *Tetraselmis sp.*, the umbo to spat was observed with a rate of 5.0 µm/day. The growth rate from '*D*' stage to spat on this species was 5.0 µm/day (Table 2. 7).

Survival rate

About 6 % of larvae were observed alive in umbo stage and about 78% of umbo settled as spat which shows a second best feed in case of settlement after attaining umbo stage. The survival upto spat stage was 4.5 % of total larvae (Fig. 2. 5).

No. of days for settlement

The spat settled on 15th day, after the initial heavy mortality till umbo stage. Initial settlement occurred on day 13.

Larvae fed with *Dunaliella salina*

Growth

The growth was considerably low in the initial stage when fed with *Dunaliella salina*. The observed mean size of umbo was 122 x 12 µm, the growth increased from umbo stage onwards. This shows *Dunaliella salina*, (9

µm size) was not good in initial stage but gains much size from umbo stage onwards. The observed mean size of spat was 307.4 x 288 µm. Fig. 2. 10 illustrate the mean size of larvae at different stages when fed with this species.

Fig. 2. 10. Mean size of larvae when fed with *Dunaliella salina*

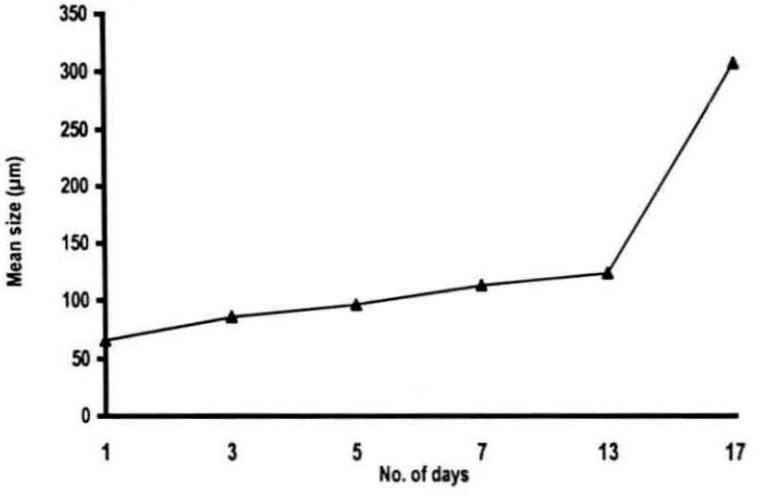


Table 2. 8. Mean growth rate and survival rate of spat

Stage	Mean growth rate (µm/day)
D – umbo	5.0 ± 0.2
umbo-spat	6.6 ± 0.5
D - spat	5.1 ± 0.5
Settling started (day)	13
Settling completed (day)	13
Rate of spat production (%)	1.7

Growth rate

The initial growth rate was 5.0 µm/day (*D* shape-spat). Thereafter, it increased significantly, till settlement, with 6.6 µm/day (umbo-spat). The growth rate from '*D*' to spat was 5.1 µm/day after settlement on 17th day (Table 2. 8).

Survival rate

Only 3 % of the larvae attained umbo stage. This shows a poor performance of this feed on larval rearing initially. However, 45.3% of umbo settled, which was higher than the umbo fed on *Dicrateria inornata* (41.5%). The survival of spat was observed with 1.36% of the total larvae (Fig. 2. 5).

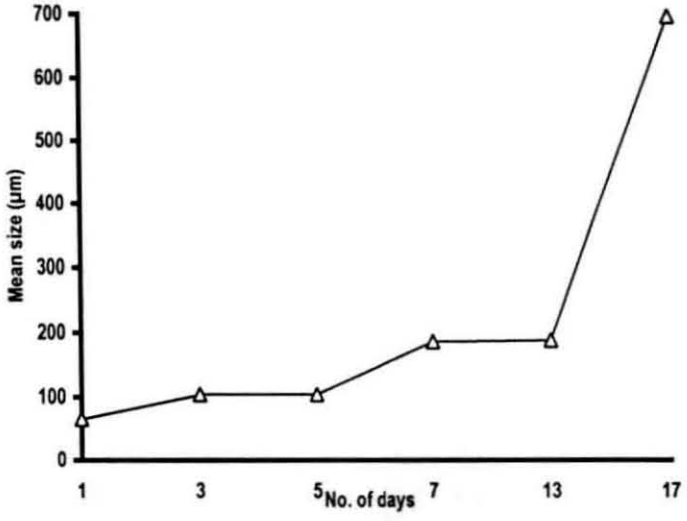
No. of days for settlement

As the larval density was considerably low in umbo stage, the development thereafter was high. The survived umbo was settled on 14th day uniformly. This was clearly indicated in clearance as well as ingestion rate on this species.

Larvae fed with *Isochrysis galbana*

As this species used as standard diet in the present study, the result obtained in the previous experiments at cell density 5 x 10^3 cells/ml considered here for the evaluation, in terms growth, survival and growth rate. The mean size of larvae at all developmental stages is represented in Fig. 2 .11.

Fig. 2. 11. Mean size of larvae when fed *Isochrysis galbana*



The growth rate (Table 2. 9) during *D* to umbo, umbo to spat and overall growth rate on analysis showed significant difference between the treatments (P≥ 0.5) and the order of performance based on the results obtained was *Nannochloropsis salina* > *Isochrysis galbana* > *Chaetoceros calcitrans* > *Tetraselmis gracilis* > *Dicrateria inornata* > *Dunaliella salina*. The mean size of larvae at different stages is represented Table 2. 10, when fed with uni algal diets.

Table 2. 9. ANOVA. Larvae fed with micro algal species alone

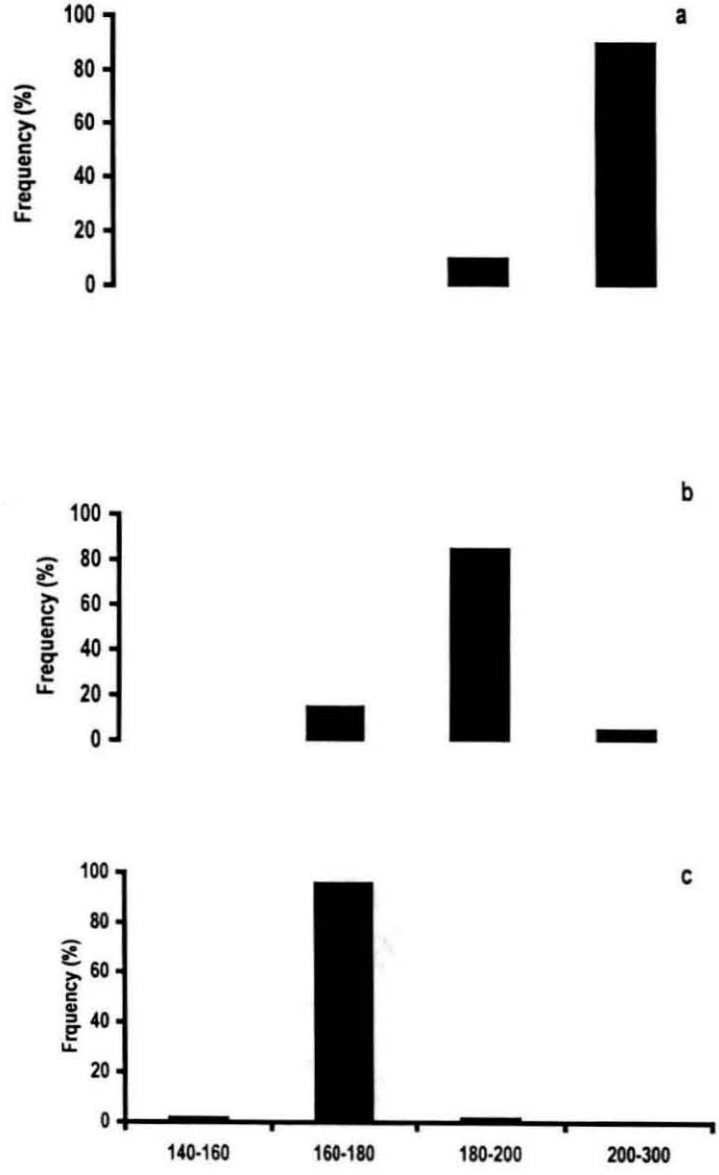
Analysis of Variance					
Source	**Sum-of-Squares**	**df**	**Mean-Square**	**F-ratio**	**P**
CONC	1571660.800	5	314332.160	181.359	0.000
DAYS	9516538.667	5	1903307.733	1098.145	0.000
CONC*DAYS	3836130.133	25	153445.205	88.533	0.000

Table 2. 10. Mean size of larvae during development when fed with monodiets

	Mean size of larvae (anterior posterior measurement) µm					
Algae → **Age of culture ↓**	*N. s*	*I. g*	*C.c*	*D. i*	*D. s*	*T. g*
3	94.4	94.4	94.4	94.0	94.0	94.0
7	147.6	154.0	146.0	124.0	124.0	124.0
10	222.0	162.0	152.0	128.0	128.0	134.0
13	342.4	188	160.0	148.0	160.0	159.0
17	656.0	673	172.0	454.0	307.0	300.0

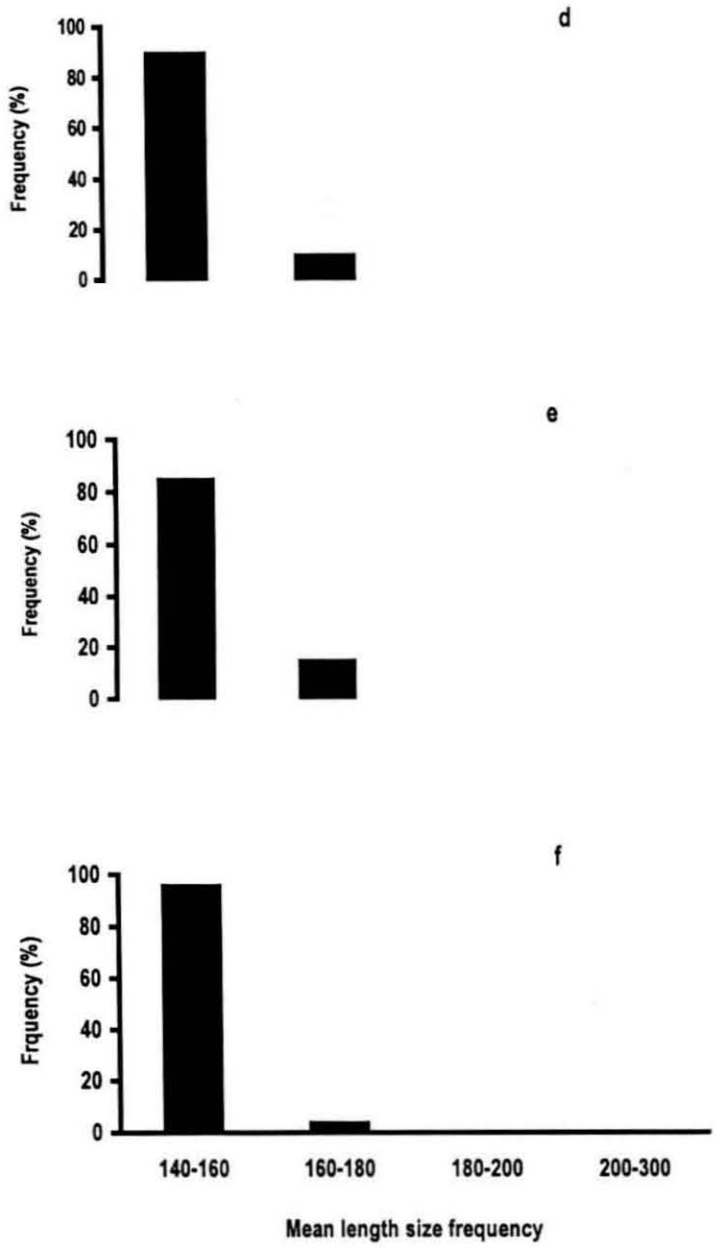
The mean size frequency (Fig. 2. 12 and 2. 13) of larva when fed with mono algal diets showed a high percentage in 200 – 300 µm range. The 95 % of spat settled were in the above range, when fed with *N. salina*. The second highest of 85 % in size frequency of 180 – 200 µm were in the spat settled, on feed *Isochrysis galbana*. Most of the spat settled in other algal feed, such as *Dicrateria sp., T. gracilis* and *Dunaliella salina* were in the size range 140 –160 µm.

Fig. 2. 12. Mean size frequency distribution of spat settled when fed with mono algal diet



a. *Nannochloropsis salina*, b. *Isochrysis galbana*, c. *Chaetoceros calcitrans*.

Fig. 2. 13. Mean size frequency distribution of spat settled when fed with mono algal diet



d. ***Dicrateria inornata***, e. ***Dunaliella salina***, f. ***Tetraselmis gracilis.***

2. 3. 2. Larval rearing with combination of two algal species

Combination *of Isochrysis galbana* and *Nannochloropsis salina*

Growth

The shell growth of larvae at various stages is illustrated in Fig 2. 14. Mean growth of larvae was significantly ($P < 0.01$) influenced by this algal combination. This combination promoted the highest larval growth during umbo stage, which was marginally higher than other nine combinations tested in the present study. Among the umbo, mean size of 104.8 x 91.2 µm was uniform. The growth of larvae was low from umbo stage as it depending on survival of umbo in later developmental stage.

Growth rate

Mean growth rate of umbo ('*D*" to umbo) during development was 8.8 µm/day and from umbo to spat, it was 19.9 µm/day. Mean growth rate of spat after settlement on 15th day was 16.2 µm/ day (Table 2.11).

Survival rate

About 14% of larvae were alive on day 7 of umbo stage. The spat settled were 78.5 % of umbo with an overall survival of 11%. A high survival rate was noticed when larvae were fed with this combination of *Nannochloropsis salina* and *Isochrysis galbana* from umbo stage onwards. About 66% and 80 % of umbo were settled when fed with monodiets.

However, survival of spat when fed alone was 32 % and 12.8 % respectively (Fig 2. 24 and 2. 25).

Fig. 2. 14. Mean size of larvae when fed with *Isochrysis galbana* and *Nannochloropsis salina*

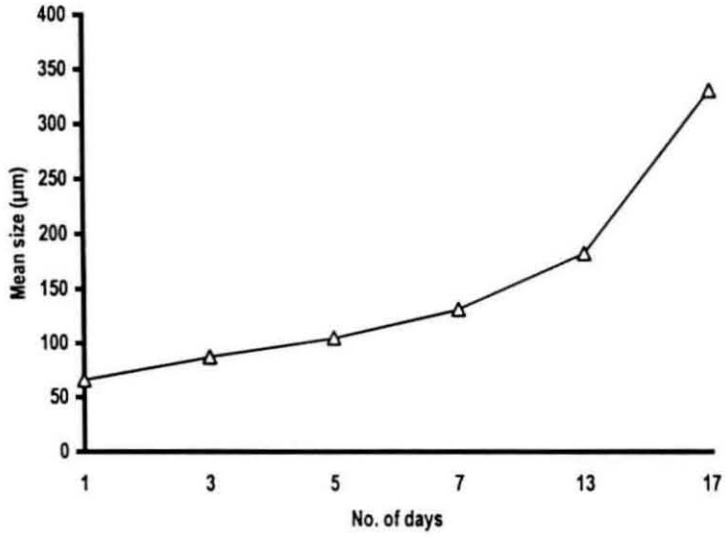


Fig. 2. 15. Mean size of larvae when fed with *Dunaliella salina* and *Nannochloropsis salina*

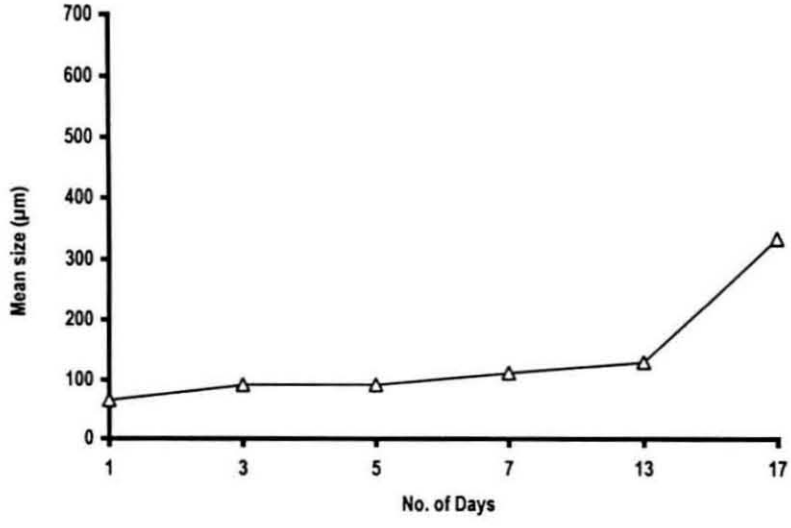


Table 2. 11. Mean growth rate and survival of spat

Stage	Mean growth rate (µm/day)
D – umbo	8.8 ± 0.2
umbo-spat	19.9 ± 0.3
D - spat	16.2 ± 0.5
Settling started (day)	10
Settling completed (day)	12
Rate of spat production (%)	11

No. of days for settlement

Even the combination diet resulted in comparatively low survival rate as well as growth rate of larvae, influenced an early settlement than any other micro algal combinations tried in the present study. The first settlement were noticed on 10th day after fertilization with a high percentage of 69% and completely settled on 12th day. Thus in terms of days for settlement, it shows very high significance, same as when *Nannochloropsis salina* was fed alone.

Combination of *Dunaliella salina* and *Nannochloropsis salina*

Growth

This algal combination promoted a low growth in early larval stages with a mean umbo size of 128 x 117 µm on 9th day after fertilization. The spat measured with a mean size of 334.4 x 284 µm (Fig. 2. 15), which is higher than spat reared on combination of *Isochrysis galbana* and *Nannochloropsis salina*. This is due to low survival of umbo in the present combination.

Growth rate

Growth rate of umbo was 6.8 µm/day. The observed growth rate from umbo to spat was 25.8 µm/day (Table 2. 12). The growth rate from '*D*' stage to spat was 15.7 µm/day.

Survival rate

About 8% of '*D*' larvae developed into umbo and 50 % settled as spat when fed with this algal combination (Fig 2. 24 and 2. 25). The survival rate of spat was a low 4.15%. Survival of umbo, fed with *Dunaliella salina* alone was lower than the larva fed with this combination, where as *Nannochloropsis salina* produced a high survival rate.

Table 2. 12. Mean growth rate and survival rate of spat

Stage	Mean growth rate (µm/day)
D – umbo	6.8 ± 0.5
umbo-spat	25.8 ± 0.5
D - spat	15.7 ± 0.4
Settling started (day)	12
Settling completed (day)	13
Rate of spat production (%)	4.2

No. of days for settlement

First settlement of spat, when larvae fed with this combination were observed on 12th day and complete settlement was seen on 13th day. As mentioned in survival conditions, *Nannochloropsis salina* influenced various stages of larval development and spat settlement, than on *Dunaliella salina*.

Combination of *Tetraselmis gracilis* and *Nannochloropsis salina*

Growth

Mean growth of larvae during various stages is presented in Figure 2. 16. The observed mean size of umbo was 112 x 107.2 µm on 7th day. Low mean size during early umbo was recorded when fed with this combination. However, spat measured a mean size of 257.3 x 225.4 µm. This combination showed a poor performance in growth pattern, compared to other combination of algae.

Growth rate

Growth rate from '*D*' to umbo in the present combination was 7.4 µm/day and it increased from umbo to spat with a mean of 20.7 µm/day. Mean growth rate from '*D*' to spat was 11.1 µm/day (Table 1. 13). Growth rate of spat when fed with combination of *Tetraselmis gracilis* (5.0 µm/day, when alone) and *Nannochloropsis salina* (19.8 µm/day) was less than when fed above species alone.

Survival rate

A low survival of 4.2 % was recorded in the umbo stage. About 75% of umbo settled as spat with an overall survival rate of 3.15% (Fig. 2. 24 and 2. 25). When larvae were fed with both species alone as mentioned in previous section, the survival rate of umbo as well as spat were significantly high in case of *Nannochloropsis salina* and moderate in *Tetraselmis gracilis*. This proves that in early stages from *D*- shape to umbo, the *Nannochloropsis*

salina alone or in combination influences maximum survival of larvae as well as spat settlement.

No. of days for settlement

The combination of *Tetraselmis gracilis* and *Nannochloropsis salina* shows first settlement on 12th day and completed on 13th day.

Table 2. 13. Mean growth rate and survival rate of spat

Stage	Mean growth rate (µm/day)
D – umbo	7.4 ± 0.4
umbo-spat	20.7 ± 0.4
D - spat	11.1 ± 0.5
Settling started (day)	12
Settling completed (day)	13
Rate of spat production (%)	3.2

Fig. 2. 16. Mean size of larvae when fed with *Tetraselmis gracilis* and *Nannochloropsis salina*

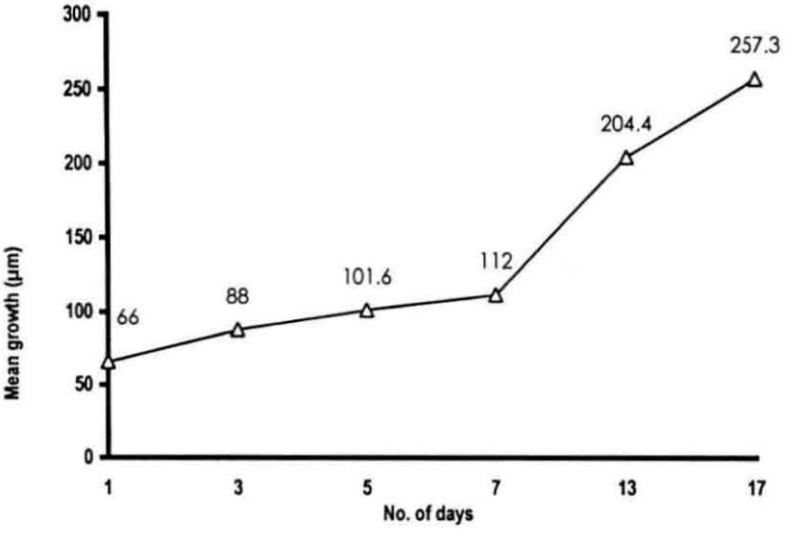
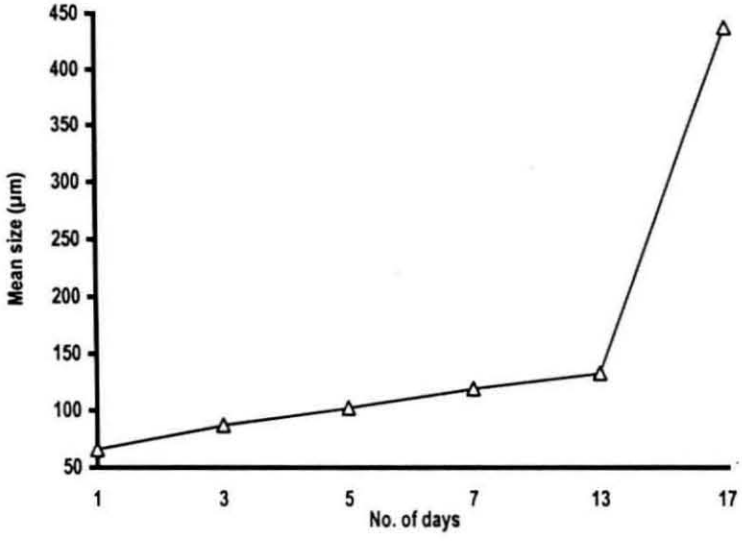


Fig. 2. 17. Mean size of larvae at various stages when fed with *Dicrateria inornata* and *Nannochloropsis salina*



Combination of *Dicrateria inornata* and *Nannochloropsis salina*

Growth

Figure 2. 17 shows mean size of larvae at various stages during development, when fed with this algal combination. Umbo size was 119.2 x 108 µm, pediveliger 132.8 x 123.2 µm and spat with an average size of 439 x 391.4 µm. The spat size measured in this combination was the highest among other algal combinations tested, but lower than when fed alone. The maximum and minimum size on 17^{th} day was 436 ± 2.16 µm and 391.4-± 4.26 µm.

Growth rate

Mean growth rate from '*D*' to umbo was measured 7.6 µm/day and from umbo to spat 22.1 µm/day. Mean growth rate of spat after settlement on

17th day was 18.1 µm/day (Table 2. 14). Low growth rate was observed in all stages when *Dicrateria inornata* fed alone than in combination. Growth rate of Umbo, when fed on combination of *Dicrateria* and *Nannochloropsis salina*, showed a higher growth rate than when fed with *Dicrateria* alone. The growth rate observed in this species was higher than any other species composition. More algal consumption was observed during this stage, especially *Dicrateria inornata* in late umbo stage, when fed in combination.

Table 2. 14. Mean growth rate and survival rate of spat

Stage	Mean growth rate (µm/day)
D – umbo	7.6 ± 0.1
umbo-spat	22.1 ± 0.2
D - spat	18.1 ± 0.5
Settling started (day)	12
Settling completed (day)	16
Rate of spat production (%)	4.2

Survival rate

About 4.8% of '*D*' stage larvae reached the umbo stage and 87.4% of umbo was settled as spat with a total of 4.2 % on 17th day. This combination performs a high survival rate from late umbo till spat settlement, than when both species fed alone. However, in earlier stage from *D*-shape to Umbo, the survival rate was high when compared to corresponding stages when fed with *Dicrateria sp.* alone. This proves again that *N.salina* is a suitable

alternate feed to *Isochrysis galbana* during development of *D*-shape, early and post umbo stages (Fig. 2. 24 and 2. 25).

No. of days for settlement

As observed a low density of umbo, the first settlement was on 12th day and completed by 16th day itself.

Combination of *Chaetoceros calcitrans* and *Nannochloropsis salina*

Growth

Figure 2. 18 represent the growth of various larval stages when fed with this combination. *D*-shape larvae had a mean size of 86.4 x 70.4 µm, after day 1 of feed ingestion. *D*-shape larvae metamorphed into umbo on 7th day. Mean size of various stages of larvae was also less than corresponding stages when fed on other mixture algal feeds. The observed mean size of umbo is 119 x 114 µm and spat 236 x 224 µm.

Growth rate

Growth rate from *D* to umbo and from umbo to spat was low when this algal species fed alone (Table 2. 15). The growth rate from umbo to spat was very low in larvae fed with *Chaetoceros calcitrans* alone (3.78 µm/day) as compared to those fed on combination *of Chaetoceros calcitrans* and *Nannochloropsis salina* (11.56 µm/day). The growth rate of spat on 17th day was 10.02 µm/day. This value is higher than the value of spat when fed

Chaetoceros alone. Here also the *Nannochloropsis salina* proves its influence on survival in early stages of larvae.

Table 2. 15. Mean growth rate and survival rate of spat

Stage	Mean growth rate (µm/day)
D – umbo	5.2 ± 0.5
umbo-spat	9.78 ± 0.5
D - spat	10.2 ± 0.2
Settling started (day)	15
Settling completed (day)	15
Rate of spat production (%)	3

Survival rate

About 5 % of larvae were alive in umbo stage, when fed with this algal combination. The survival rate was higher than when fed *Chaetoceros* alone (3%). However, 88% of umbo settled as spat when fed alone as against 60% in combination. But the *Chaetoceros* along with *Dicrateria* or *Isochrysis* gave a performance of 74.1 % and 74. 5% respectively, while *Nannochloropsis sp* gave a survival of 87.4 % and 78.57% (Fig. 2. 24 and 2. 25).

The survival of spat on 17th day was observed to be 3% of total larvae, where it was 2.64% when fed *Chaetoceros sp* alone, but lower than *Nannochloropsis salina* (32%).

No. of days for settlement

As low survival rate was observed in this combination of feed, the larval density was low and concentration of feed regime was quite high when

compared to larval stages fed with other combinations. It was observed that larvae settled on 15th day of feeding trials.

Fig. 2.18. Mean size of larvae when fed with *Chaetoceros calcitrans* and *Nannochloropsis salina*

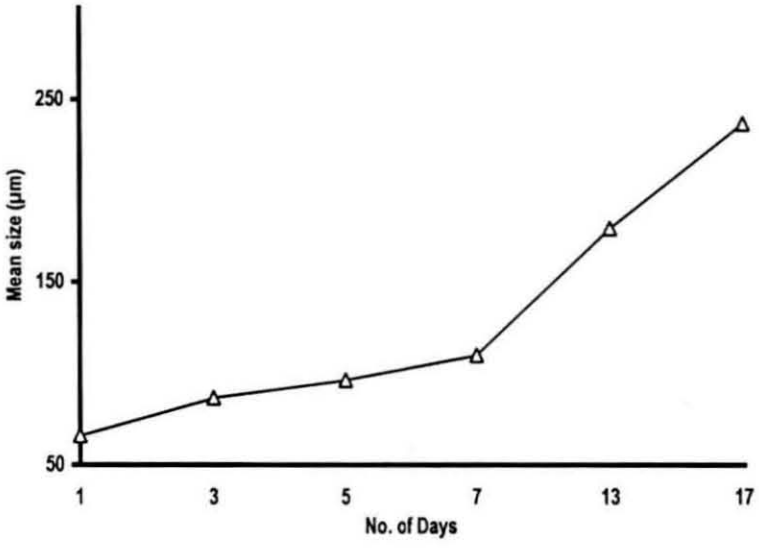
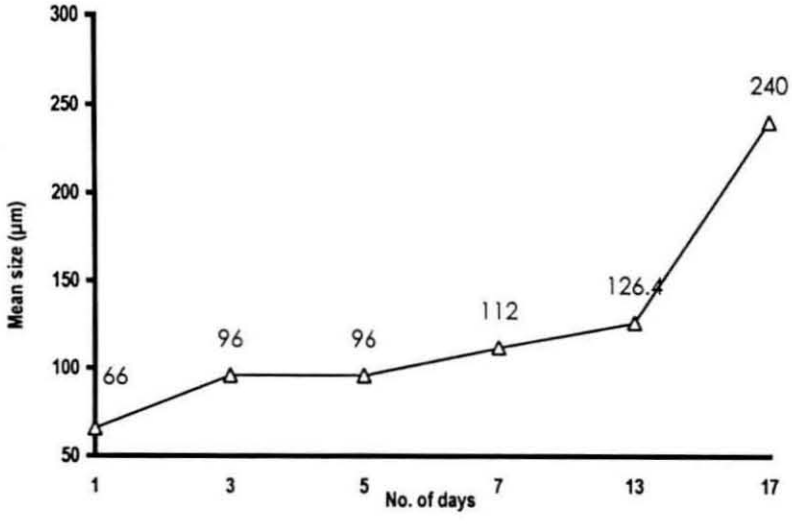


Fig. 2.19. Mean size of larvae when fed with *Isochrysis galbana* and *Tetraselmis gracilis*



Combination of *Isochrysis galbana* and *Tetraselmis gracilis*

Growth

Fig 2.19 represents mean shell length of various stages of larvae during 16 day period i.e., upto settlement. The mean size on 5th day was relatively low, 96 x 96 µm, the umbo stage was observed on 8th day with a mean size of 120.6 x 112 µm. The settled spat was with a low size of 240 x 228 µm, among all other algal combinations on 17th day.

Growth rate

As observed in growth, the growth rate was also low from '*D*' to umbo with 7.6 µm/day (Table 2. 16). From umbo to spat, it was 10.6 µm/day. The whole spat growth rate observed was very low with 10.2 µm/day among mixed diets as well as monodiets.

Table 2. 16. Mean growth rate and survival rate of spat

Stage	Mean growth rate (µm/day)
D – umbo	7.6 ± 0.2
umbo-spat	10.6 ± 0.5
D - spat	10.2 ± 0.3
Settling started (day)	15
Settling completed (day)	17
Rate of spat production (%)	4.2

Survival rate

About 5% larvae survived to umbo stage, when fed with this algal combination and 84 % of umbo settled as spat with a 4.2% of settlement (Fig. 2. 24 and 2. 25). This combination as well as both species alone, when fed in late umbo stage showed an equal survival rate and settlement. However, survival rate was low, when fed in combination during early stages. This is due to the preference of larvae on *Isochrysis galbana* (7-8 µ) over *Tetraselmis gracilis* (12-14 µ) in terms of algal size.

No. of days for settlement

First settlement of larvae was observed on 15th day (58%) and complete settlement on 17th day.

Combination of *Isochrysis galbana* and *Dicrateria inornata*

Growth

Average size of early umbo was 100 x 84 µm, which is low among algal combinations. The observed maximum mean size was 176 x 168 µm on day 6 and the spat settled 271 x 264 µm. The Figure 2. 20 represent growth of larvae at various stages during development.

Growth rate

From *D*-shape to umbo, a growth rate of 5.6 µm/day was observed and 19.0 µ m/day from umbo to spat (Table 2. 17), quite higher than when

fed with *Dicrateria inornata* alone. From umbo to spat, the growth rate was 19.0 µm/day for combination of two algal species, which was higher than the above species when fed alone. The spat growth rate observed on day 16th after feeding trial was 12.7 µm/day. This growth rate was comparatively lower than the value observed in combination of *Dicrateria sp.* and *Nannochloropsis salina* (18.1 µm/day) and combination of *Nannochloropsis sp.* and *Isochrysis sp.* (15.1 µm/day).

Table 2. 17. Mean growth rate and survival rate of spat

Stage	Mean growth rate (µm/day)
D – umbo	5.7 ± 0.2
umbo-spat	22.2 ± 0.2
D - spat	8.6 ± 0.6
Settling started (day)	13
Settling completed (day)	15
Rate of spat production (%)	4

Survival rate

The observed survival of umbo and spat in this algal combination was 4 % and 4. 15 % repectively, which was higher, when *Dicrateria* fed alone (1.65%). *Dicrateria* with *Nannochloropsis salina* also showed a high survival rate (4.15%) (Fig. 2. 24 and 2. 25).

No. of days for settlement

First settlement was observed on 15th day and rest of larvae settled next day.

Fig. 2. 20. Mean size of larvae when fed with *Isochrysis galbana* and *Dicrateria inornata*

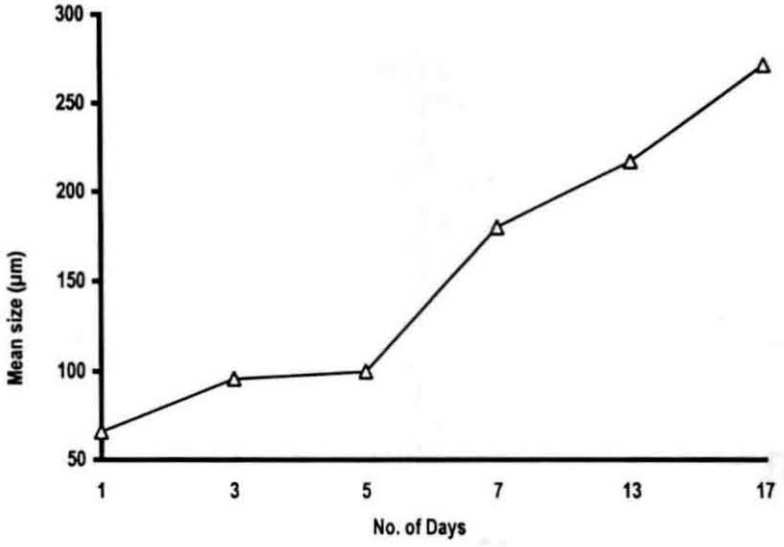
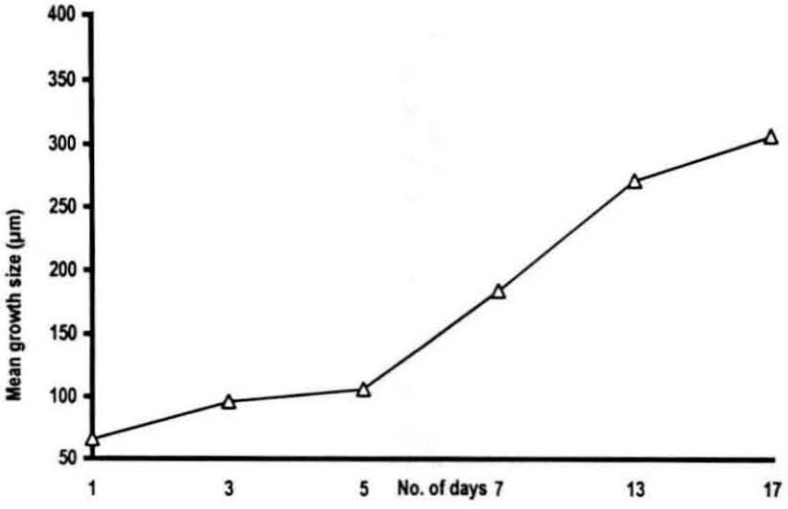


Fig. 2. 21. Mean size of larvae when fed with *Isochrysis galbana* and *Chaetoceros calcitrans*



Combination of *Isochrysis galbana* and *Chaetoceros calcitrans*

Growth

Fig 2. 21 illustrate average growth of larvae in different stages during development. The early umbo stage was with a mean size of 106.4 x 105.6 µm, which was high among all other algal combinations. But settled spat measured with a mean size of 306.4 x 296.8 µm. When larvae fed *Chaetoceros calcitrans* alone, growth was low compared to this combination.

Growth rate

Growth rate from '*D*' to umbo and umbo to spat was 5.7 µm/day and 22.2 µm/day respectively, when fed with this combination. The growth rate of umbo (2.3 µm/day) and *D* to spat (8.6 µm/day) was low, when larvae fed *Chaetoceros sp* alone. However, from '*D*' to spat stage, growth rate was high (22.2 µm/day) among other combinations Table 2.18.

Survival rate

About 74.1% of umbo was settled with a spat survival rate of 4.02 % (Fig. 2. 24 and 2. 25).

No. of days for settlement

It was observed that initial settlement on day 13 and complete settlement by day 15.

Table 2. 18. Mean growth rate and survival rate of spat

Stage	Mean growth rate (µm/day)
D – umbo	5.7 ± 0.2
umbo-spat	22.2 ± 0.2
D - spat	22.0 ± 0.3
Settling started (day)	13
Settling completed (day)	15
Rate of spat production (%)	4.0

Combination of *Dicrateria inornata* and *Chaetoceros calcitrans*

Growth

The Fig 2. 22 illustrate the mean size of various stages of larvae, when fed with this algal combination. The mean size of umbo was 144 µm x 134.4 µm on day 8 and spat 353.6 x 335.2 µm on day 16, which was above the mean size while fed *Dicrateria sp* and *Chaetoceros sp* alone. However, mean size of spat on 16th day on mixed diet was low than when fed *Dicrateria sp* alone; but high when *Chaetoceros sp* fed as monodiets.

Growth rate

The combination produced high growth rate in initial stage (Table 2. 19) than when both species fed alone. The mean growth rate from '*D*' to umbo was 9.0 µm/day in this algal combination, where it was 8.6 and 5.0 µm/day respectively when fed as monodiets. The growth rate from umbo to spat was 4.9 µm/day and 2.3 µm/day respectively. The growth rate from '*D*' to spat was 15.0 µm/day.

Table 2. 19. Mean growth rate and survival rate of spat

Stage	Mean growth rate (µm/day)
D – umbo	9.0 ± 0.2
umbo-spat	4.9 ± 0.2
D - spat	15.0 ± 0.3
Settling started (day)	14
Settling completed (day)	16
Rate of spat production (%)	3.1

Survival rate

About 4% of larvae were metamorphed into umbo and 74.5% of them settled as spat with a total survival of 3.1%. Here the combination provides maximum survival rate than when larvae fed these species alone (Fig. 2. 24 and 2. 25).

No. of days for settlement

First settlement (35%) was observed on 14^{th} day and complete settlement on 16^{th} day.

Fig. 2. 22. Mean size of larvae when fed with *Dicrateria inornata* and *Chaetoceros calcitrans*

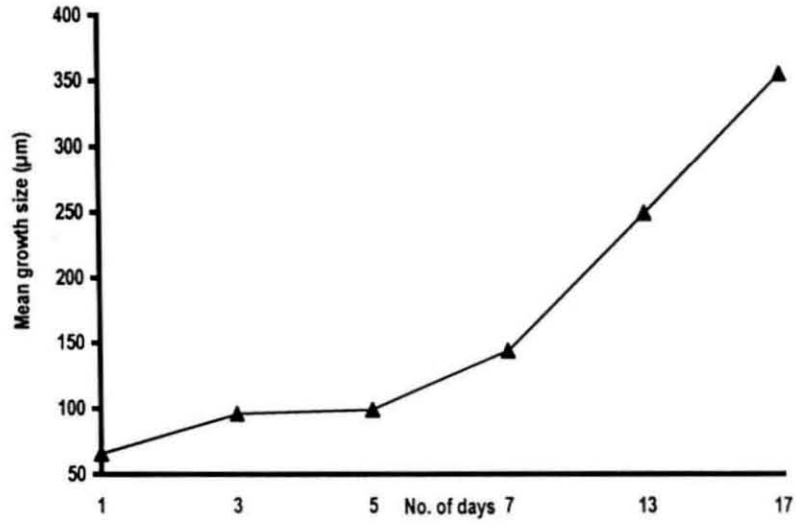
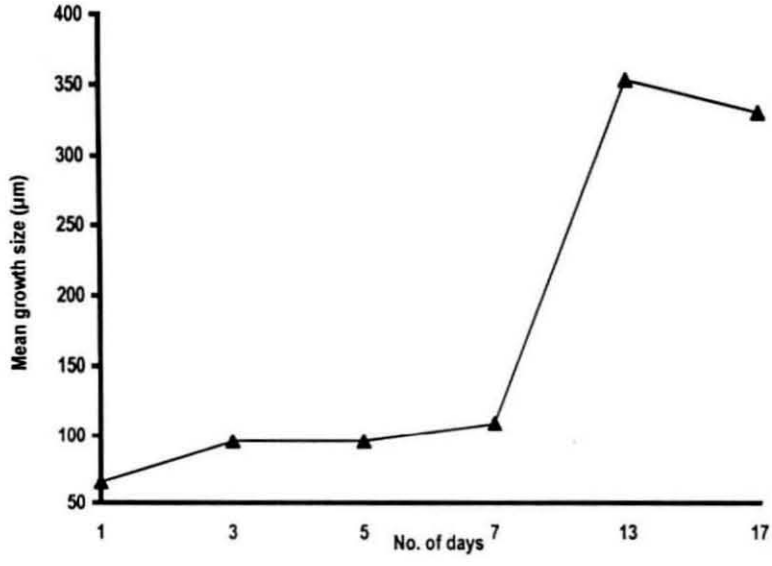


Fig. 2. 23. Mean size of larvae when fed with *Dicrateria inornata* and *Tetraselmis gracilis*



Combination of *Dicrateria inornata* and *Tetraselmis gracilis.*

Growth

The mean size of different stages of larvae when fed this algal combination is illustrated in Fig. 2. 23. The mean size of early umbo on 5th day was 108.3 x 104 µm. The mean size of spat after 17 days, when fed this combination, was 330 x 302 µm, which was high than that larvae fed with *Tetraselmis gracilis* alone. However, when *Dicrateria sp.* fed alone, the mean size of spat was slightly higher than the combination of *Dicrateria sp.* and *Tetraselmis gracilis* but lower than *T. gracilis* alone.

Growth rate

The mean growth rate from '*D*' to umbo stage was 7.1 µm/day and umbo to spat 11.0 µm/day (Table 2. 20). The growth rate was low when fed with *Tetraselmis sp* alone but higher than that of *Dicrateria sp.* The growth rate of spat in the combination was (16.5 µm/day), which was higher than larvae fed *Tetraselmis sp* (5.0 µm/day) and lower than *Dicrateria sp* (19.8 µm/day) as monodiet. The result showed increase in growth rate from umbo stage to spat, as they may have preference of *Tetraselmis sp* over *Dicrateria sp* based on cell size.

Survival rate

About 4.2% of larvae were alive in umbo stage and 85% of them settled as spat with a survival 3.6% (Fig. 2. 24 and 2. 25). The survival of

umbo in this combination was moderately high among the combinations of *Dicrateria sp.*, where it was low among other *T. gracilis* combinations.

Table 2. 20. Mean growth rate and survival rate of spat

Stage	Mean growth rate (µm/day)
D – umbo	7.1
umbo-spat	11.0
D - spat	16.5
Settling started (day)	14
Settling completed (day)	16
Rate of spat production (%)	3.6

No. of days for settlement

The initial settlement was on 14th day (33%) followed by complete spat settlement on 16th day.

The growth rate during *D*-umbo, umbo to spat and overall growth rate on analysis showed significant (P≥ 0.5) (Table 2. 21) variation between the treatments and the order of performance, when fed in mixture of two algal species, based on the results obtained is in the combinations Nannochloropsis *salina* > *Isochrysis galbana* > *Dicrateria inornata*. The combination of following species, *Tetraselmis gracilis*, *Chaetoceros calcitrans* and *Dunaliella salina* on analysis shows no significance between them (P≤ 0.05).

Table 2. 21. ANOVA. Larvae fed with combination of two algal species

Analysis of Variance					
Source	Sum-of-Squares	df	Mean-Square	F-ratio	P
CONC	253736.801	9	28192.978	33.491	0.000
DAYS	8043703.424	5	1608740.685	1911.027	0.000
CONC*DAYS	996860.084	45	22152.446	26.315	0.000

Maximum size frequency of 300 – 400 µm was observed in larvae fed with combinations of *N. salina* with *I. galbana* and *D. inornata* (95 %). The spat settled when fed with combination of *I. galbana* and *D. inornata* produced 80 % but in the size range of 260- 300 µm. the low size range of 200 –220 µm was observed in combination of *N. salina* with *C. calcitrans* (10%). The percentage of size frequencies of spat fed with different algal combinations is represented in Figure 2. 26, 2. 27 and 2. 28.

Fig. 2. 24. Percentage of survival of umbo stage and spat settled when reared in different feed regime

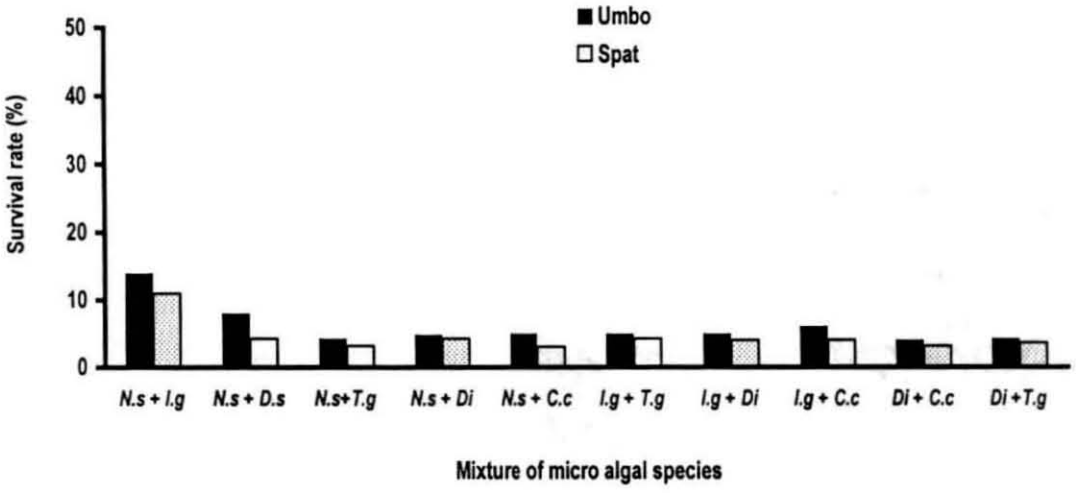


Fig. 2. 25. Survival of umbo stage and percentage of umbo settled as spat when reared in different feed regime.

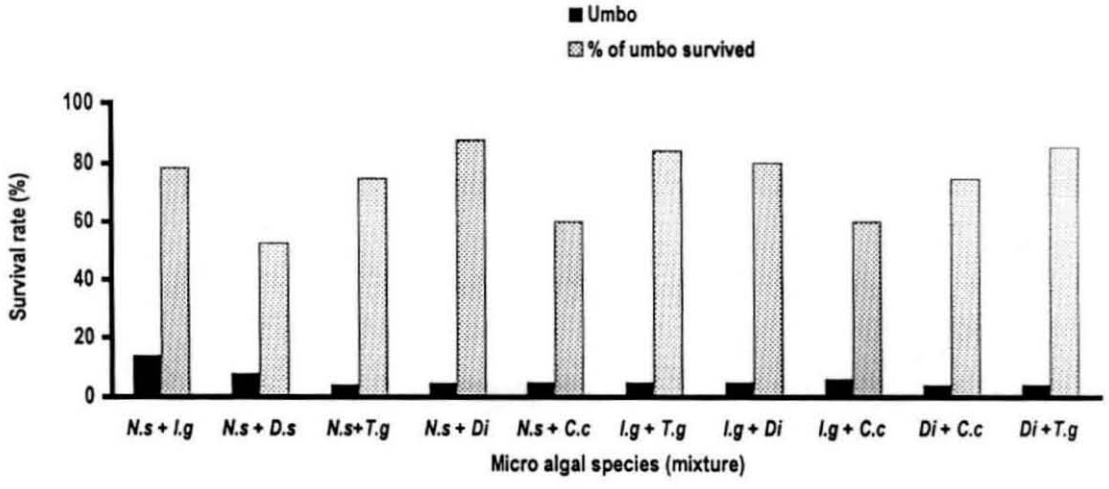
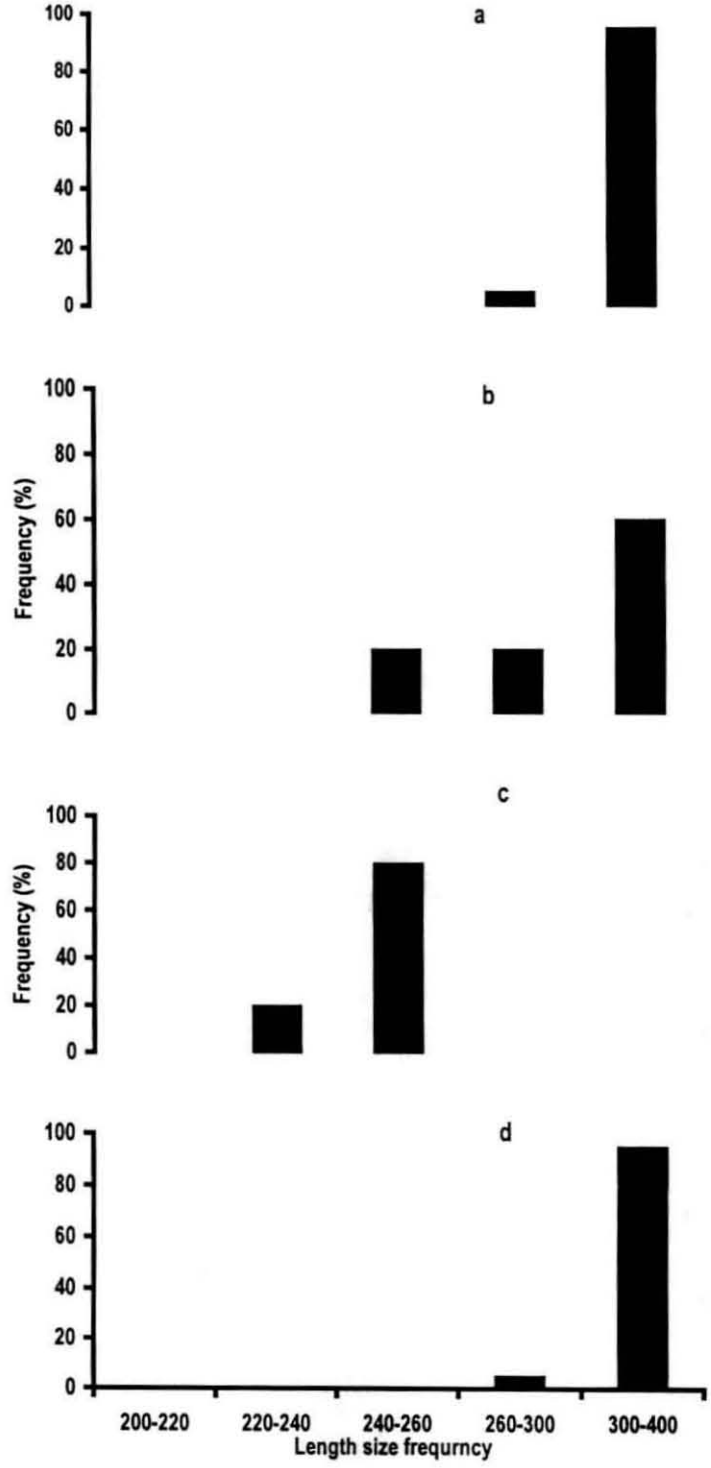
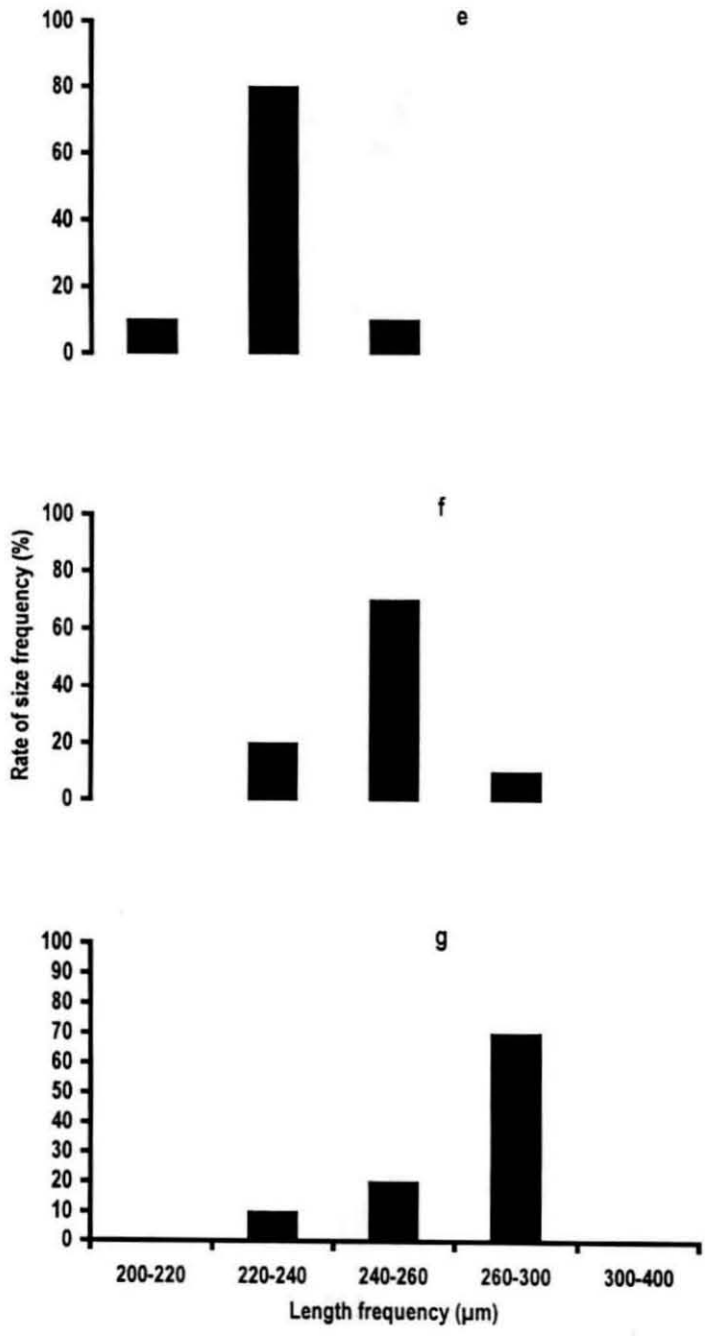


Fig. 2. 26. Mean size frequency distribution of spat when fed with mixed diets



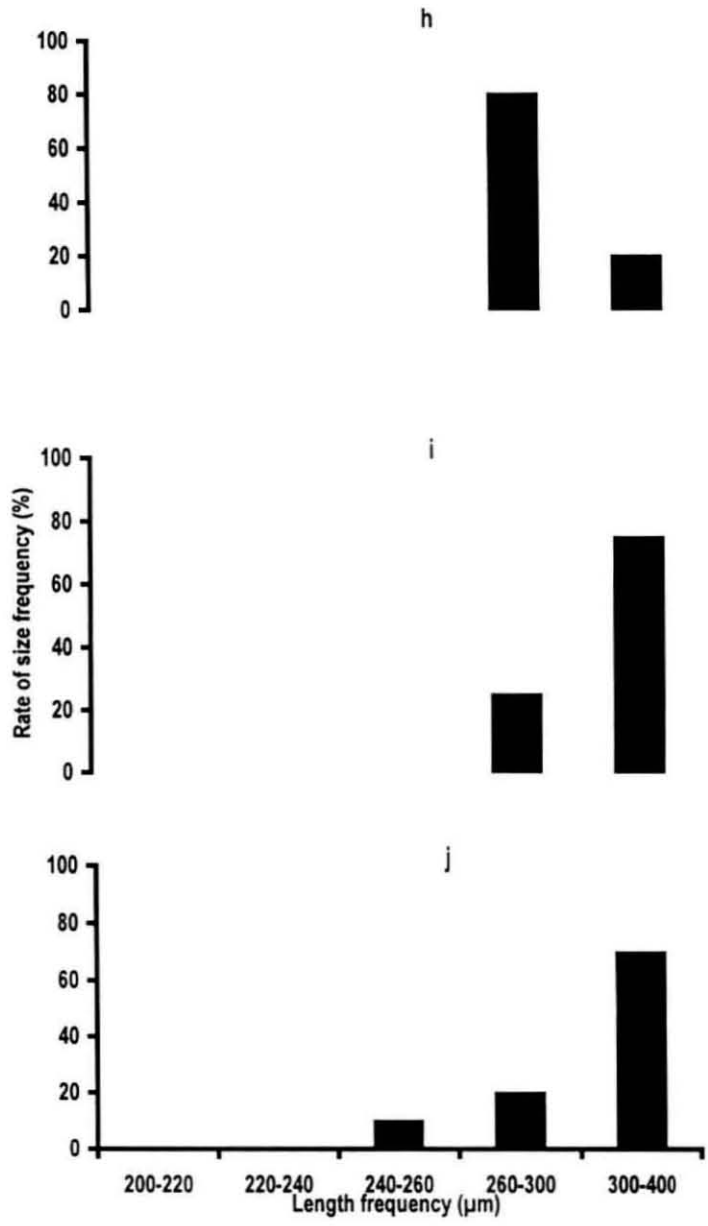
a. ***N.s +l.g***, b. ***N.s + D.s***, c. ***N.s + T.g***, d. ***N.s + Di.***

Fig. 2. 27. Mean size frequency distribution of larvae fed with mixed diets



e. *N.s* + *C.c*, f. *l.g* + *T.g*, g. *l.g* + *Di*

Fig. 2. 28. Mean size frequency distribution of larvae fed with mixed diets



h. *I.g* + *C.c*, i. *Di* + *C.c*, j. *Di* + *T.g*

Abbreviations. ***I.g* = *Isochrysis galbana*; *C.c* = *Chaetoceros calcitrans*; *D.i* = *Dicrateria inornata*; *T.g* = *Tetraselmis gracilis*; *D.s*= *Dunaliella salina* and *N. s*= *Nanochlorpsis salina***

2. 4. Index of relative nutritional value of micro algal diets

To compare the nutritional quality of micro algae used in the present investigations, an index of nutritional value was calculated based on growth rate of larvae reared on different diets in above rearing experiments. The index value is determined using the following expression.

$$\text{Index of relative nutritional value of the test species} = \frac{\text{Larval growth regression rate of the test algal species}}{\text{Larval growth regression rate of standard algal species}}$$

Isochrysis galbana, which was used in the experiment I as control algal species is considered here as standard algal species. The growth rate of *Isochrysis galbana* is 0.92 log μm/day (Log value). The observed relative index value of micro algal species tested in the experiments is summarized in Table 2. 22.

The relative index value of only one algal species greater than 1 is *Nannochloropsis salina* (1.02). *Chaetoceros calcitrans* (1.01) also showed a similar index value. All other micro algal species have relative index value less than 1. The order of performance of algal species in terms of relative index value in species is as *Dicrateria inornata* (0.73), *Tetraselmis gracilis* (0.73) and *Dunaliella salina* (0.73). Even, the *Chaetoceros calcitrans* have a relative index value 1.01, the other factors such as survival rate, growth and

age of spat settlement is also considered further, in order to determine the best algal species, with *Nannochloropsis salina*.

In combination of *Isochrysis galbana* with *Nannochloropsis salina*, the observed relative index value (1.24) is higher than, when *Nannochloropsis salina* was fed alone (1.02). The other algal combination with *Nannochloropsis salina* produced relative index value greater than 1. The order of their performance, based on relative nutritional index value, is as follows: *Nannochloropsis salina* + *Dicrateria inornata* (1.35) > *Nannochloropsis salina* + *Dunaliella salina* (1.27) > *Nannochloropsis salina* + *Tetraselmis gracilis* (1.16) > *Nannochloropsis salina* + *Chaetoceros calcitrans* (1.08). The relative nutritional index value of algal species other than the combinations of *Nannochloropsis salina* were also calculated.

The micro algal combination of *Nannochloropsis salina* with *Isochrysis galbana* is considered as standard species (1.17). Thus the combination of *Isochrysis galbana* with *Tetraselmis gracilis*, *Dicrateria inornata* and *Chaetoceros calcitrans* is observed with an index value 0.86, 0.94 and 1.0 respectively. The combination of *Dicrateria inornata* with *Chaetoceros calcitrans* and *Tetraselmis gracilis* is observed with a value 1.0 and 1.03 respectively. Even the last two algal combinations show a value higher than 1, larval survival and spat production is very much lower than the test algal species.

Table 2. 22. Index of relative nutritional value of micro algae

Algal diet	Growth rate regression (log µm/day) Standard diet	Test diet	Relative index
I. g	0.92		
N. s		0.94	1.02
C. c		0.93	1.01
D. i		0.69	0.73
T. g		0.69	0.73
D. s		0.69	0.73
Mixture of diets.			
N. s + I. g		1.17	1.24
N. s + D. s		1.41	1.27
N. s + T. g		107	1.16
N. s + D.i		1.25	1.35
N. s + C.c		1.0	1.08
I. g + N. s	1.17		
I. g + T. g		1.01	0.86
I. g + D. i		1.10	0.94
I. g + C.c		1.17	1.00
D. i + C. c		1.17	1.00
D. i + T. g		1.21	1.03

I.g =Isochrysis galbana, N. s = Nannochloropsis salina, C.c = Chaetoceros calcitrans, D.i = Dicrateria inornata, T.g = Tetraselmis gracilis

Experiment IV

Comparison of *Nannochloropsis salina* with *Isochrysis galbana* when reared at different cell densities

Growth

The Figure 2. 29 represents mean size of larvae at various stages, when fed with four different cell concentrations of *Nannochloropsis salina* and *Isochrysis galbana*. The mean size of umbo observed was 121.8 x 120 µm at 5 x 10^3 cells/ml concentration of *N. salina*, while *I. galbana* was 108.9 x 104 µm. The mean size of pediveliger was 188 x 180 µm in case of *I. galbana* and 255 x 240 µm in *N. salina* at same concentration. The settled spat was with a mean size of 693 x 622 µm and 660 x 644 µm respectively in *Isochrysis galbana* and *Nannochloropsis salina*.

At 3 x 10^3 cells/ml concentration, the umbo was with a mean size of 151 x 148 µm in *N. salina* while it was 160 x 144 µm in *I. galbana*. The mean size of pediveliger was 164.8 x 156 µm and 166.6 x 154 µm respectively in *N. salina* and *I. galbana*. The settled spat was with a mean size 540 µm in *N. salina*, where it was 643.2 x 602 µm for *I. galbana* (Fig. 2. 29).

At 4 x 10^3 cells/ml concentration, the mean size of umbo was 157.6 x 148 µm in *N. salina* and 149.6 x 146.5 µm in *I. galbana*. The pediveliger was with a size of 165.6 x 160 µm and 198.4 x 172 µm in *N. salina* and *I. galbana*

respectively. The pediveliger settled as spat was with a high mean size 1 mm x 990 µm. However, it was very low in case of *N. salina* with a mean low size 584.6 x 556 µm. This is due to survival and development of larvae from umbo stage onwards during the period.

At 6 x 10^3 cells/ml concentration, the umbo mean size measured as 148.8 x 144 µm in both algal diets. There was a change in during the pediveliger stage with a mean size of 163.2 x 156 µm and 180.8 x 172 µm respectively in *Nannochloropsis salina* and *Isochrysis galbana*.

Growth rate

The growth rate was 37.12, 32.96, 37.97 and 32.85 µm/day respectively at 3000, 4000, 5000 and 6000 cells/ml concentrations in *Nannochloropsis salina*, while it was 37.12, 61.6, 39 and 32.8 µm/day in larvae fed with *Isochrysis galbana*.

Survival rate

There was an inverse relationship, in case of larvae fed with *Isochrysis galbana*, with cell concentration, where an increase in concentration showed a chance of low survival. However, a positive correlation, in larvae fed with *N. salina,* where an increase in algal concentration gave maximum survival at 5 x 10^3 cells/ml concentration. About 39.3 % of larvae survived till umbo stage, when *N. salina* fed at a

density 5 x 10^3 cells/ml (Fig. 2. 30). About 83 % of umbo was settled with a total spat settlement rate of 32.8 %. 32 % of larvae were settled and survived when *I. galbana* fed at a 4000-cell/ml concentration.

No. of days for settlement

The first settlement of spat was in *N. salina* when fed at 5000 cell/ml concentration on 9^{th} day (78%) and whole spat settlement on 11^{th} day. In other cell densities, first settlement was on day 10 in 3000, day 9 in 4000 (48%) and day 10 in 6000 cells/ml concentrations.

The initial settlement in case of *I. galbana* was observed in 4000-cells/ml concentration (45%) on 13^{th} day and whole spat settlement on 14^{th} day. In 5000 cells/ml, it was on 13^{th} day of experiment (42 %) and whole spat settlement on 15^{th} day.

Fig. 2. 29. Larvae fed with *I. galbana* and *N. salina* at different concentrations and in combination

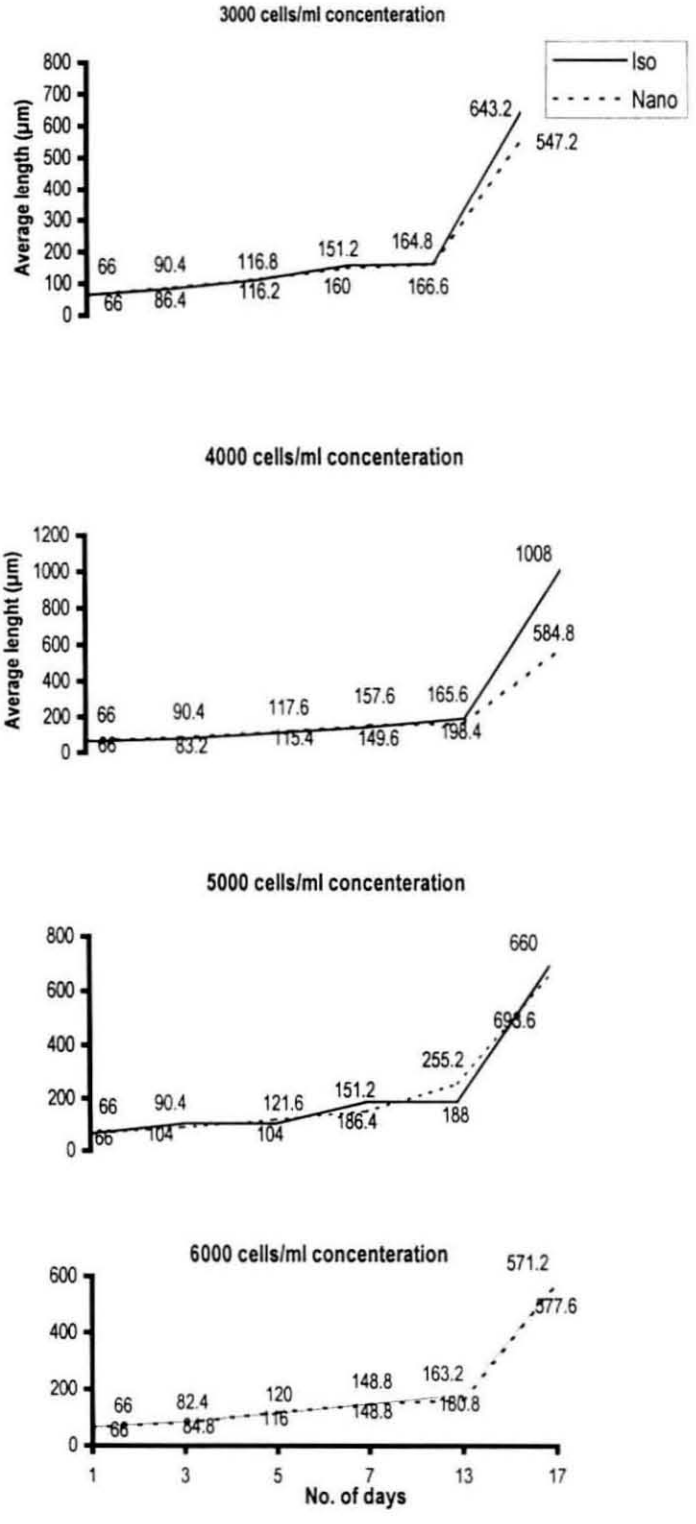


Table 2. 23. Growth rate of spat fed with micro algae both in combination and alone

Algal diet	Growth rate mm/day)
I. g	0.16
N. s	0.10
C. c	0.12
D. i	0.01
T. g	0.09
D. s	0.12
Mixture of diets.	
N. s + I. g	0.16
N. s + D. s	0.10
N. s + T. g	0.12
N. s + D.i	0.09
N. s + C.c	0.14
I. g + T. g	0.14
I. g + D. i	0.12
I. g + C.c	0.14
D. i + C. c	0.08
D. i + T. g	0.09

I.g =Isochrysis galbana, N. s = Nannochloropsis salina, C.c = Chaetoceros calcitrans, D.i = Dicrateria inornata, T.g = Tetraselmis gracilis

II. Juvenile/spat

The spat reared with different micro algal diets both singly and in combination of two algal species attained almost uniform pattern of growth. The observed anterior posterior measurement of juveniles reared in six micro algal diets show a minimum variation between them in terms of size. The growth rate is presented in Table 2. 23. The result obtained in this experiment is carried out as the basis for the evaluation of various micro algal feed in the following chapter III, where filtration rate and clearance rate are discussed in detail to conclude the biological observation made during the above experiments.

2. 5. Discussion

In spite of a number of different theories about the nature of food for bivalves, it is established that primary source of food consists of phytoplankton (Ukeles, 1971). The nutritional requirements of marine fishes and prawns can be met by using artificial diets. In contrast, phytoplankton still remains the main food source for bivalve larvae and most algal species promotes growth. In most existing hatcheries, the larvae are fed with one or several micro algal species, which are generally selected, as they promote acceptable larval growth and are easy to grow.

Many workers have been reported that one of the vital factors in producing large number of bivalves is the type and quality of algal food for

their rearing (Rhodes and Lander, 1973). Ukeles (1980) and Roessler (1990) reported that larvae have very specific food requirement, which if not fulfilled, will result in abnormal growth, disease or mortality. Many short term studies were done in bivalves on micro algal nutritional aspects (Thompson and Bayne, 1972, 1974; Widdow 1978 a, b; Griffiths and King 1979; Griffiths, 1980; Navarro and Winter, 1982; Winter, 1978; Winter *et al.,* 1984; Hawkins and Bayne, 1992). The commercial hatchery culture of bivalve, thus require a supply of live micro algal food cells of high nutritional value.

The food value of a wide range of algae has been assessed for the culture of oysters (Walne, 1970; Tenore and Dunstan, 1973; Enright *et. al.,* 1986). A number of experiments have been carried out to determine the suitability of micro algae for the commercial culture of other bivalves too. In most studies, larval size in mean length at different stages was taken as criteria to evaluate the algal feed regime. A few works has been done on relative growth rate (Bayne, 1965). Early conclusion was that larval forms make considerable use of flagellates such as *Isochrysis galbana, Pyramimonas grossi, Pavlova sppl,* while the same exhibit poor growth with nonmotile species such as in post settlement phase.

The suitability of algae as an adequate food for bivalves is associated with a series of factors such as cell size, digestibility of cell wall and nutritional value or toxicity due to secondary metabolites. Differences in these factors, which determine algal quality, affect survival, growth,

metamorphosis and setting rates. De Pauw (1964, 1981) reviewed from bibliographic data, the nutritive value of about 43 micro algal species, belonging to nine classes as food for oysters (*Ostrea edulis* and *Crassostrea gigas*), clams (*Venerupis pullastra* and *Mercenaria mercenaria*) and mussels (*Mytilus edulis*). According to De Pauw, *Dicrateria inornata*, *Isochrysis galbana, Pseudoisochrysis paradoxa, Tetraselmis chuii,* and *Chaetoceros calcitrans* are considered as excellent feed. Most bivalve species efficiently retain particles between 2 and 7 µm size. The *Crassostrea virginica* adults may filter particles between 3 and12 µm sizes. However, larvae use 1- 2 µm size particles. Walne (1974) reports that optimal density varies with size of the algae offered as food ranging from 4 X 10^3 cells/ml with bigger cells to 500 X 10^3 cells/ml with smaller cells. Epifanio (1979) while supporting Walne studied the effect of 15 diets with different mixture of algae on the growth of *Crassostrea virginica* and *Mercenaria mercenaria* and also concluded that growth was not correlated with gross chemical composition but with presence or absence of a particular algal species in the diet offered. The best diet according to them was *Isochrysis galbana* and *Tetraselmis paradoxa* in combination.

Anuradhakrishnan (1993) reported that the mixed diet of *Isochrysis galbana* with *Tetraselmis gracilis* was good for growth and survival of the oyster, *Pinctada fucata*. A few studies were reported on nutrition in clams. Teresa *et al.*(2000) reported significant difference in survival, shell growth

and glycogen concentrations of finger nail clams fed with different diets, thus implying that same diets were better than others.

The relative food value of algal diets was examined in the clam, *Tapes semidecussata* (Laing *et al.*, 1987). Walne (1970) found very high growth rate of *Mercenaria mercenaria* fed with *Skeletonema costatum*, low growth with *Pavlova tricornutum* and very little growth with *Chlamydomonas coccoides*. Epifanio (1979) reported that *Tetraselmis sueciaca* promoted moderate growth of *Mercenaria mercenaria* juveniles. A good survival and moderate growth are observed in Atlantic surf clam, *Spisula solidissima*, larvae fed on *Isochrysis galbana,* but survival was low in other micro algal species. Other bivalve species fed with these diets show similar growth and growth rates (Pierson, 1983; Laing and Millican, 1986 and Enright *et al.*, 1986).

In the present study, *Isochrysis galbana,* when fed at concentration 5 x 10^3 cells/ml promoted better result in terms of growth and survival, where as *Nannochloropsis salina* in low density 4 x 10^3 cells/ml. As the larvae did not progress and develop in other algal species *Dunaliella salina* and *Tetraselmis gracilis,* with a high protein level, the reason is to be considered as the size of food particles. The low growth rate in umbo stage was due to low consumption of *Dunaliella salina* in early period when fed alone. Survival of umbo, fed with *Dunaliella salina* alone was lower than the larva fed with this combination, where *Nannochloropsis salina* produced a high survival

rate. Clearance rate and ingestion rate of this *Dunaliella sp.* or *Tetraselmis sp.* was low, when fed alone and in combination with *Nannochloropsis salina.* The results of the present study showed that *Nannochloropsis salina* promoted good growth and produced maximum settlement. This shows a combination of *Nannochloropsis salina* with any other algal species influences survival of larvae in early stages prior to settlement.

Regarding growth rate, the suitable dietary algal species were different in various stages of the same bivalve species (O'connor *et al.*, 1992). Wada (1973) noted that *Pavlova lutheri* (19 µm/day) is more suitable diet than *Chaetoceros calcitrans* for larvae of Pearl oyster (12 µm/day), however, the *Chaetoceros calcitrans* showed best result in post umbo and settling rate. Okauchi (1990) found that *I. galbana* is considered as ideal diet for pearl oyster juveniles than in larvae. The same is reported in *Pinctada fucata* (Numaguchi, 2000), while working with five algal species on pearl oyster juveniles. But not, mentioned on larval stages.

Although it is noted that effect of larval density and algal ration influences the growth and survival of larvae (Tanka *et al.,* 1970; Helm and Millican, 1977; Lu and Blake, 1996), the present study was conducted for the evaluation of micro algal species only, while keeping larval density 1 larva/ml. This is considered for the evaluation of micro algal species at individual level to determine biochemical variations in developmental stages.

Regarding the algal combinations, the *Isochrysis galbana* with *Nannochloropsis salina,* used in the present study represented good growth and development in various life stages among algal combinations. The relative index on nutritional value of various algal diets used in the present study reveals the above algal combination with a relative index of 1.24, as the best-feed regime. The combination of *Nannochloropsis salina,* with other algae used in the study also shown better result than other algal combinations.

Rene *et al.* (2001) reported the efficiency of *Tetraselmis suecica* for *Crassostrea gigas* larvae, umbo, as a component diet. But, not recommended as a monospecific diet and also at early stage of development. The same with *I. galbana,* showed success in *Ostrea edulis* larvae of 160-195 µm (Helm, 1977) and on umbo-spat in *Crassostrea* larvae, with *I. galbana* and *Chaetoceros calcitrans* (Utting and Spencer, 1991). But no work is dealt with early *D* shape larvae to umbo development, where the size of food particles forms a factor. Thus in the present investigation, *N. salina* (2 µm) shows high growth, survival *and* larval settlement in combination with flagellate *Isochrysis galbana.*

Regarding spat settlement, the present study on this clam, *Paphia malabarica,* larvae fed with *N. salina* and the combination of same with *Isochrysis galbana* and *Dicrateria inornata* showed an early settlement on day 9, 10 and 13 respectively. Percentage of setting has rarely been used as

criteria for appraisal of algal quality. No literature was reported on period of settlement, while working on efficiency of various micro algal species. However, somewhat similar reports were made on pearl oyster larval settlement with initial on15th day and final settlement on 17th day (Anuradhakrishnan, 1993) and 13th day in clam *Venerupis ruditapes,* (Albentosa *et al.,* 1999), while rearing with *I. galbana* as feed. Review of literatures gives only the parameters growth, growth rate and survival rate were considered as the criteria for micro algal evaluation as feed in larval development of molluscs.

It could be concluded that, on management point of view, the micro alga *Nannochloropsis salina* is the ideal feed in early larval development and the combination of the same with any other flagellates will yield high survival and spat production.

Chapter 3

3. Filtration and clearance rate of *Paphia malabarica* larvae fed with different diets

3.1. Introduction

Apart from biological view on performance of various algal diets, the availability and cosumability of suitable algal cells were also noticed as an important aspect of bivalve nutrition. Many workers recorded filtration rate as a function of body and used for qualitative estimation of larval nutrition. This was measured based on either direct or indirect methods. The indirect method like, estimation of growth at different feed levels (Davis and Guillard, 1958; Rhodes and Landers, 1973) or rate of clearance of particles from the medium (Wilson, 1980). The direct method involves like radioactive labelled micro algae (Walne, 1965; Ukeles and Sweeney, 1969; Lohrenz and Taylor, 1987) or observation with Epifluorescent microscopy (Lucas and Ranger, 1983). Most of the works in literature were concerned with feeding and food supply and the way larvae develop under certain feed regimes (Rajesh *et al.*, 2001; Riisgard, 1988; Sandra *et al.*, 1985), but very few works are available concerning feeding and filtration rates of molluscan larvae under different regimes. Gerdes (1983) studied on feeding behaviour of larvae and adults of the pacific oyster *Crassostrea gigas*. Davis and Guillard (1958) noted on growth of bivalve larvae with a mixed diet and also the constituent diet separately (Walne and Spencer, 1968; Helm, 1977). Great differences in food value between algal species has been reported by several authors (Cole, 1937; Walne, 1963, 1965), but not, how two algal species, which are found to be good food organisms in bivalve larvae, than when fed as

monospecific cultures (Davis and Guillard, 1958; Walne, 1970). Albentosa *et al.* (1996), evaluated live micro algal diet for the seed culture of *Ruditapes decussatus* using physiological and biochemical parameters, but not mentioned on feeding behaviour. Authors used acceptability, digestibility and growth as criteria to evaluate nutritional efficiency of micro algae.

Lucas and Rangel (1983) detected, first larval feeding from *D* larvae onwards in *Crassostrea gigas*, using the epifluorescence microscopy. Larval response to food added, revealed 80% of *D* shape *C. gigas* larvae started feeding (Babinchak and Ukeles, 1979). However, it was not mentioned about anything on micro algal species preferred. All the previous studies were mainly on *Isochrysis galbana* and a few on *Pavlova sp.* and *Chaetoceros sp.*

In the present study on *Paphia malabarica* larvae at different stages, six micro algal species were tested both singly and in combination as explained in previous Chapter. This chapter reveals a brief study on filtration rate, clearance rate and consumption of algal cells during the experiments.

3. 2. Material and Methods

3. 2. 1. Filtration Rate

Larvae from the same brood were reared according to methods mentioned in Chapter 2. *Isochrysis galbana* at different cell concentrations was tested in the first experiment. In one tank, algal cells without larvae

were kept in order to test the possible multiplication of algal cells in the medium during the experiment. The initial cell concentrations of algal cells in each experimental tank were noted. The mean temperature, pH and salinity during the experiment are 29 ± 1.5°C, 8.0-8.3 and 31 ± 1 $^{o}/_{oo}$ respectively. The samples were taken and fixed with 5% diluted formalin and later counted the number of algal cells in each ml using a haemocytometer. The samples were also taken for counting concentration of cells after 24 hours prior to water exchange daily, as final cell concentration. The filtration rate was calculated using the following formulae (Coughlan, 1969) in *D* shape, Umbo and settled spat,

$$\text{Filtration rate} = \frac{\text{Log } C_1 - \text{Log } C_2}{t} \times \frac{V}{d},$$

Where, C_1 = Initial cell concentrations (ml)

C_2 = Final cell concentrations (ml)

t = Duration of experiment (Hrs) i.e. , as 24 hrs

V= volume of the medium (ml) and

d = density of larvae.

3. 2. 2. Clearance rate

Total number of algal cells removed by larvae in the medium at the end of 24 hours per ml was counted. Clearance rate is also expressed as % of the initial algal cell concentrations per ml of volume.

The same method was adopted in the rest of the experiments (mono diets, combination of two algal diets and in experiment on comparison of *Isochrysis* galbana with *Nannochloropsis* salina and combination of these two algal diets.

3. 3. Results

3. 3. 1. Filtration rate

a) Larvae fed with *Isochrysis galbana* at different cell densities

The mean filtration rate of larvae has been expressed as the average of the filtration of larvae at all stages of development. The filtration rate showed decrease with increasing cell concentrations (Table 3.1). The observed mean filtration rate in concentrations 3, 4, 5 and 6 x 10^3 cell/ml was 0.005, 0.003, 0.01 and 0.004 ml/lar/hr respectively (Fig 3.1). The filtration rate also showed reduction as the cell concentration decreased, with an increase in size of larvae during development (Fig 3.2).

Table. 3. 1. Mean filtration rate of larvae in various stages at different concentrations of *Isochrysis galbana*

Cell density (x 10^3 cells/ml) ↓	Filtration rate (ml/larva/hr)		
	D shape larvae	umbo	settled spat
3	0.002	0.01	0.005
4	0.005	0.004	0.003
5	0.01	0.01	0.01
6	0.004	0.004	0.004

b) Larvae fed with monoalgal diet

The filtration rate in *D* shape larvae, umbo and settled spat varied with different micro algal species (Table 3. 2). The mean filtration rate of larvae has been expressed as the average of filtration of larvae at all stages, when fed with micro algal diets alone (Fig 3. 3).

Fig. 3. 1. Mean filtration of larvae fed with different cell densities of *Isochrysis galbana*

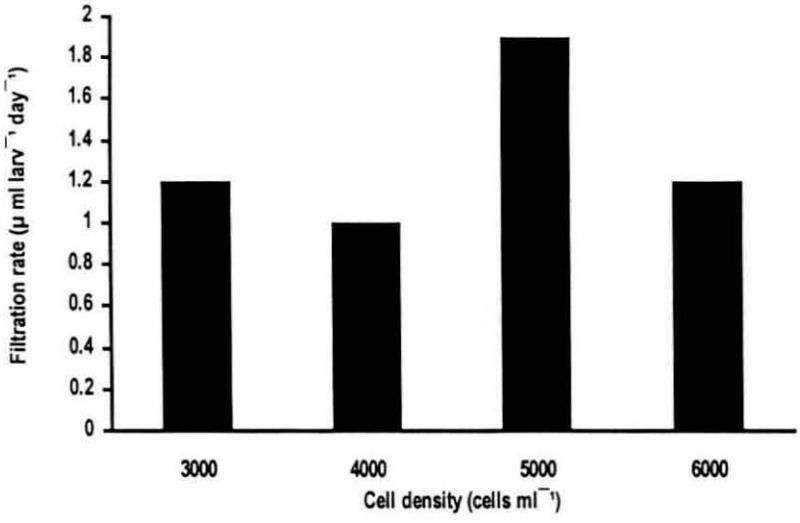


Fig. 3. 2. Mean filtration rate of larvae during development in different cell densities of *Isochrysis galbana*

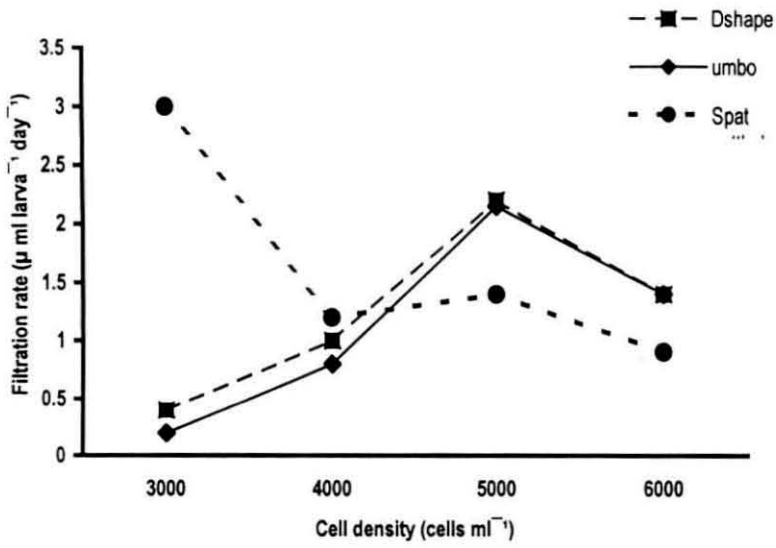


Table 3. 2. Mean filtration rate of clam larvae when fed with mono algal diets

Algal species ↓	Filtration rate ($ml^{-1}larva^{-1}hr^{-1}$)		
	D shape	umbo	settled spat
Nanochloropsis salina	0.04	0.04	0.03
Isochrysis galbana	0.03	0.08	0.03
Chaetoceros calcitrans	0.03	0.01	0.02
Dicrateria inornata	0.01	0.01	0.02
Dunaliella salina	0.01	0.01	0.01
Tetraselmis gracilis	0.01	0.01	0.01

Fig. 3. 3. Mean filtration rate of larvae when fed with mono algal diets

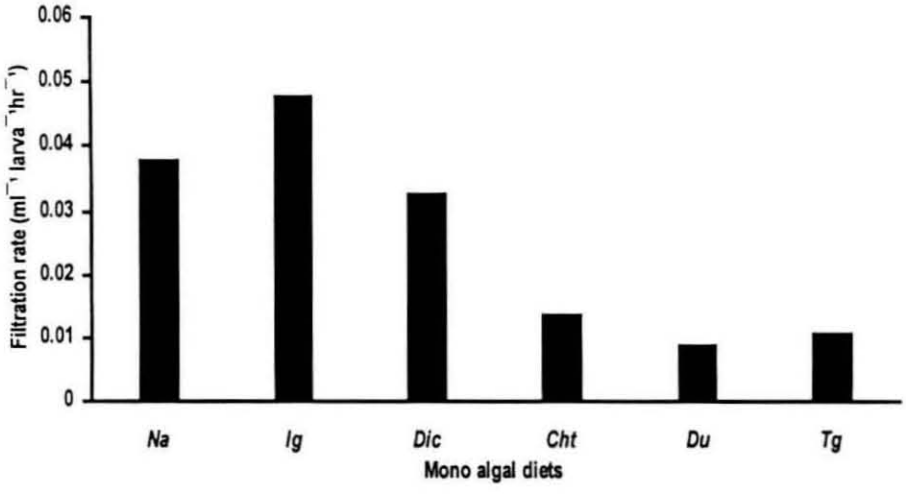


Table 3. 3. One-way ANOVA on filtration rate between mono algal diets

Source	Sum of squares	df	Mean square	F- ratio	P
Treatment (between)	0.008	5	0.002	18.987	0.000
Error	0.003	30	0.000		

The observed mean filtration rate of larvae on one-way analysis of variance shows significance at 1 % level (Table 3. 3).

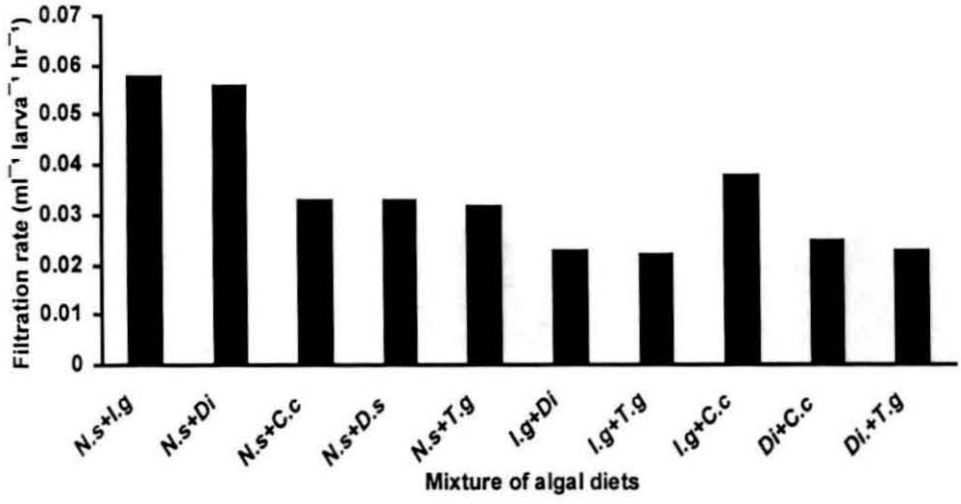
c) When fed with combination of two micro algal diets

Here the filtration rate varied unlike the monoalgal diets. The size of food particles had influenced on filtration activity. The size of micro algae used in the present study in the ascending order was *Nanochloropsis salina* (2-3 µm), *Isochrysis galbana* (7-8 µm), *Dicrateria inornata* (8-9 µm), *Dunaliella salina* (9-11 µm), *Chaetoceros calcitrans* (11-12 µm) and *Tetraselmis gracilis* (12-15 µm). The mean filtration rate of larvae when fed with combination of algal species is represented in Figure 3. 4. Larvae preferred the low size food particles initially followed by large particles, during its development (Table 3. 4).

Table 3. 4. Mean filtration rate of clam larvae when fed with combination of two algal species

Algal species ↓	Filtration rate ($ml^{-1}larva^{-1}hr^{-1}$)		
	D shape	umbo	settled spat
N.s+I.g	0.041	0.041	0.042
N.s+D.i	0.037	0.04	0.038
N.s+C.c	0.019	0.019	0.019
N.s+D.s	0.013	0.013	0.013
N.s+T.g	0.013	0.013	0.013
I.g+D.i	0.022	0.023	0.023
I.g+T.g	0.023	0.023	0.006
I.g+C.c	0.021	0.023	0.023
D.i+C.c	0.023	0.023	0.021
D.i+T.g	0.018	0.017	0.019

Fig. 3. 4. Mean filtration rate of larvae when fed with mixture of algal diets



Abbreviations: *N.s = Nannochloropsis salina, I.g = Isochrysis galbana, D.i = Dicrateria inornata, C.c = Chaetoceros calcitrans, D.s = Dunaliella salina* and *T.g = Tetraselmis gracilis.*

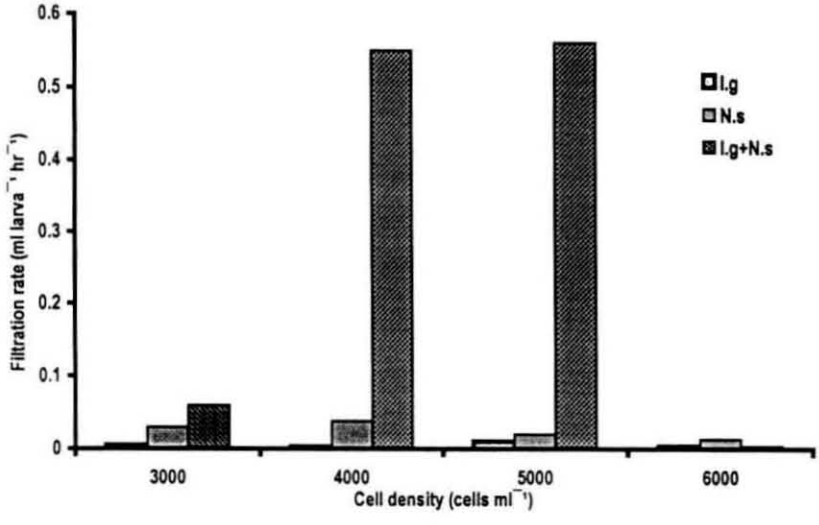
d) Comparison between *Nanochloropsis salina* and *Isochrysis galbana* at different cell densities

The mean filtration rate of larvae in these diets is represented in Table 3. 5. The combination of two algal species did not show much variation from the average filtration rate of both species, when fed alone. The mean filtration rate of all larval stages together in these three groups is represented in Figure 3. 5. The mean filtration rate, on statistical analysis, did not show significant variation between the treatments ($P<0.001$).

Table 3. 5. Mean filtration rate of larvae fed with both as single diet and in combination

Algal species ↓	Filtration rate ($ml^{-1}larva^{-1}hr^{-1}$)		
	D shape	umbo	settled spat
Nannochloropsis salina	0.05	0.04	0.04
Isochrysis galbana	0.04	0.07	0.05
N. salina + *I. galbana*	0.05	0.05	0.06

Fig. 3. 5. The mean filtration rate of larvae fed with ***N. salina***, ***I. galbana*** and in combination



3. 3. 2. Clearance rate

a) Larvae when fed with *Isochrysis galbana*

The clearance rate enhanced with increasing larval size at all concentrations. The observed clearance rates in *D* shape larvae, umbo and settled spat are summarised in Table 3. 6. The mean clearance of algal cells by larvae has been expressed as the average clearance of algal cells at all three stages (Fig. 3. 6). The observed mean clearance rate in cell densities 3, 4, 5 and 6 x 10^3 cells/ml were 40, 25.3, 38 and 20% respectively. One-way analysis of variance (Table 3. 7) showed that clearance rate is significantly ($P \geq 0.05$) influenced by cell concentrations.

b) When fed with mono algal diets

The highest clearance rate was found in larvae fed with *N. salina* and *D. inornata* (8.55 and 8.44 x 10^3 cells respectively). The mean clearances of algal cells in *D* larvae, umbo and spat when fed with various micro algal diets is summarised in Table 3.8. The observed mean clearance rate of micro algal species in the whole development of larvae is presented in Figure 3. 7.

The size of the food particle is a criteria for the clearance of algal cells by larvae in various stages. The mean clearance rate against the size of the micro algae used in the present study shows that the larvae prefer low cell size at initial stages, as they are easy to filter (Fig. 3. 8). The clearance of algal cells by larvae has also been expressed as the average clearance of algal cells (Table 3. 8).

Table. 3. 6. Mean clearance rate of larvae in various stages fed with *Isochrysis galbana*

Cell density (x 10^3 cells/ml) ↓	Clearance of cells (x 10^3 cells)		
	D shape larvae	umbo	settled spat
3	0.4	0.2	3
4	1	0.8	1.2
5	2.2	2.15	1.4
6	1.4	1.4	0.9

Table 3. 7. One-way ANOVA on filtration rate between mono algal diets

Source	Sum of squares	df	Mean square	F- ratio	P
Treatment (between)	0.008	5	0.002	18.987	0.000
Error	0.003	30	0.000		

Fig. 3. 6. Mean clearance rate of *I. galbana* at different cell densities

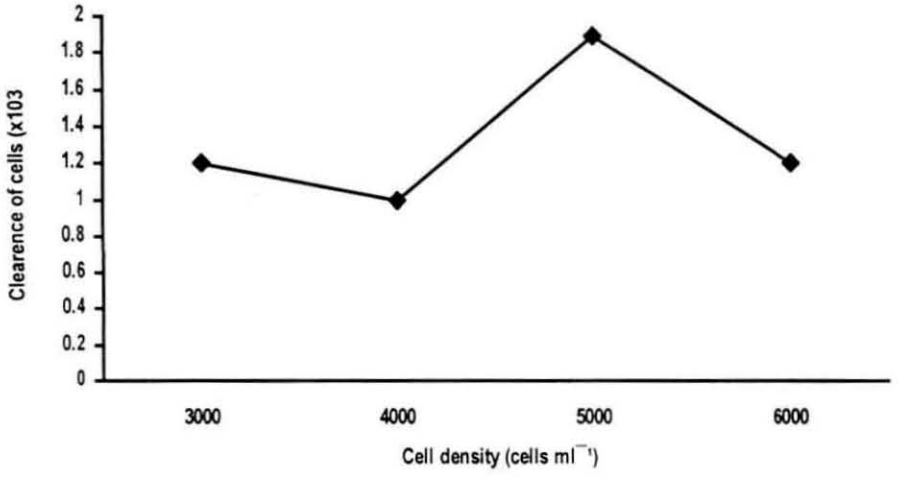
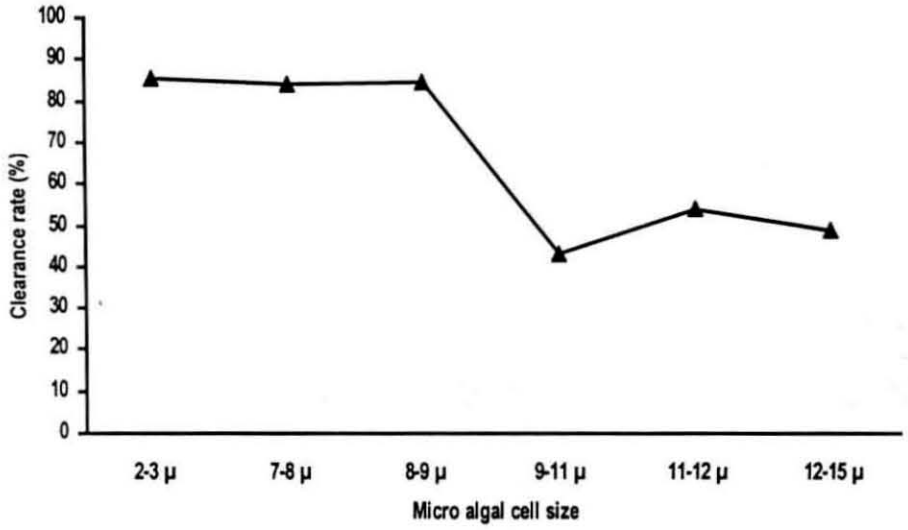


Fig. 3. 8. Mean clearance rate of various micro algal cells depending on cell size



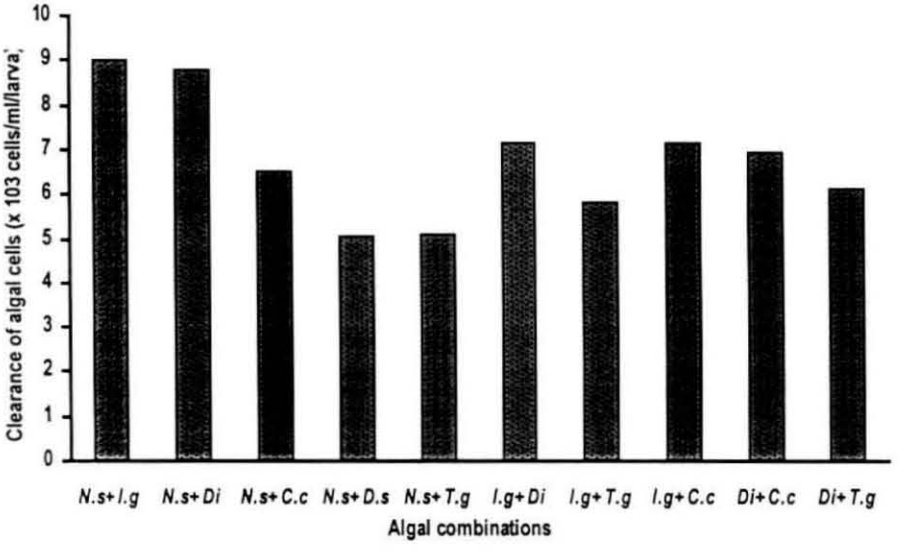
c) Larvae fed with combination of algal diets

In combination, high consumption of algal cells was observed *in N. salina* with *I. galbana* (9 x10^3cells/ml) and *N. salina* with *D. inornata* (8.8 x10^3cells/ml). The mean value in different stages of development, when fed with algal combination is mentioned in Table 3. 9. The mean clearance of algal cells by larvae altogether after settlement is presented in Figure 3. 9. The mean rate of clearance of algal cells against the initial concentration is also been expressed in Table 3. 9.

Table 3. 9. Mean clearance of algal cells when fed with combination of two micro algae

Algal combinations	Clearance of algal cells (x 10^3 cells/ml/larva)				
	D larvae	umbo	settled spat	Mean clearance of algal cells	Clearance rate (%)
N.s + I.g	9.00	9.00	9.02	9.00	90
N.s + D.i	8.70	8.90	8.80	8.80	88
N.s + C.c	6.46	6.50	6.60	6.52	65.2
N.s + D.s	5.00	5.00	5.20	5.06	50.6
N.s + T.g	5.20	5.00	5.10	5.10	51.0
I.g + D.i	7.10	7.20	7.20	7.15	71.5
I.g + T.g	7.20	7.30	3.00	5.83	58.6
I.g + C.c	6.90	7.30	7.30	7.16	71.6
D.i + C.c	7.00	6.90	6.90	6.96	69.6
D.i + T.g	5.80	6.50	6.50	6.13	61.3

Fig. 3. 9. Mean clearance of cells by larvae when fed with combination of two algal species



Abbreviations: *I.g* = *Isochrysis galbana*, *N.s* = *Nannochloropsis salina*, *D.i* = *Dicrateria inornata*, *D.s* = *Dunaliella salina*, *C.c* = *Chaetoceros calcitrans* and *T.g* = *Tetraselmis gracilis*.

Table 3. 10. Mean filtration rate of algal cells by spat fed with micro algae

Algal species	Filtration rate (cells/ ml/larva/hr) (log) settled spat
I.g	0.035
N.s	0.037
D.i	0.033
C.c	0.014
D.s	0.013
T.g	0.016
Mixture of algal diets.	
N.s + *I.g*	0.058
N.s + *D.i*	0.056
N.s + *C.c*	0.033
N.s + *D.s*	0.033
N.s + *T.g*	0.032
I.g + *D.i*	0.023
I.g + *T.g*	0.022
I.g + *C.c*	0.038
D.i + *C.c*	0.025
D.i + *T.g*	0.023

Abbreviations: *I.g* = *Isochrysis galbana*, *N.s* = *Nannochloropsis salina*, *D.i* = *Dicrateria inornata*, *D.s* = *Dunaliella salina*, *C.c* = *Chaetoceros calcitrans* and *T.g* = *Tetraselmis gracilis*.

Table 3. 11. Mean clearance of algal cells by spat in different life stages

Algal species	Clearance of cells (x 10^3 cells/ml)	Mean clearance rate (%)
I.g	8.60	86.0
N.s	8.70	87.0
D.i	8.44	84.4
C.c	5.43	54.4
D.s	5.19	51.9
T.g	6.00	60.0
Mixture of algal diets.		
N.s + I.g	9.60	96.0
N.s + D.i	9.55	95.5
N.s + C.c	8.90	89.0
N.s + D.s	8.40	84.0
N.s + T.g	8.30	83.0
I.g + D.i	7.20	72.0
I.g + T.g	7.10	71.0
I.g + C.c	8.80	88.0
D.i + C.c	7.55	75.5
D.i + T.g	7.24	72.4

Abbreviations: *I.g = Isochrysis galbana, N.s = Nannochloropsis salina, D.i = Dicrateria inornata, D.s = Dunaliella salina, C.c = Chaetoceros calcitrans* and *T.g = Tetraselmis gracilis.*

3. 5. Discussion

Results of the present investigation indicate that larvae start feeding from '*D*' shape, as reported in all bivalve larvae (Lucas and Rangel, 1983). The filtration rate is influenced by algal cell concentration. The highest filtration rate is observed in cell density 5 x 10^3 cell/ml (0.01ml/larv/hr) of *I. galbana*, when fed at different cell concentrations. However, *N. salina* was filtered out at a rate of 0.04 ml/larva/hr in the same density. Filtration rate of larvae in different algal cell concentration showed that larvae are able to regulate feeding activity according to the food concentration in the medium. The same is comparable to *I. galbana*, when fed to *Crassostrea gigas* at different densities (Gerdes, 1983).

The clearance rate obtained in other species is comparable to the present study. The values obtained in *Argopecten irradians* larvae are 4 - 5 µl/hr, when fed with *I. galbana* (Gallager and Mann, 1989). The similar rate is also reported in other species, such as 4.5 – 5.5 µl/hr for *Mercenaria mercenaria* (Riisgard, 1988), *Ruditapes philippinarum* (Albentosa *et. al.*, 1999) in the cell density 5 –6 x 10^3 cells/ml.

Regarding filtration rate to food particle size, *N. salina* being 2-3 µm indicated a mean filtration rate of 0.04ml/larva/hr, which showed high value in the present investigation, when fed alone. The mixture of the same species with *I. galbana* showed to be the best among all tested algal combinations and produced highest growth rate and suggests it to the ideal feed to develop larvae of good quality. In contrast to the feeding

activity of clam juvenile, filtration rate of larvae did not increase continuously with increasing body size. When fed the same concentration of cells to larvae and juveniles, filtration rate showed a similar pattern from late umbo stage onwards. However, the highest filtration rate was observed in settled spat during developmental stages.

The results obtained in the present investigations is comparable to earlier works on particle retention efficiency of suspension feeding bivalves, as in *Mya arenaria* (Flemming *et al.*, 1978) and *Venerupis pullsatra* (Albentosa *et al.*, 1999), which filtered out particle size below 4 µm. Blake (1961) found that *Nannochloris atomus* (2 x 2 µm) were cleared by *Mya arenaria*, with about half the rate of much bigger algae such as *I. galbana* and *Chaetoceros calcitrans*.

Clearance rate increased with increasing larval and juvenile size at same cell concentrations. High clearance of *I. galbana* cells is found in low cell concentrations in juveniles (3x 10^3 cells/ml). It is about 80% in 4x 10^3 cells/ml and 40 % in 6x 10^3 cells/ml.

Regarding the clearance rate of algal cells, obtained clearance rate is high in *D* shape larvae, when fed algae below 4 µm size. The *N. salina* showed best performance with a high rate of 91, 82 and 83% in *D* larvae, umbo and settled spat respectively. The mean clearance rate of other micro algae is written in order of merit as *Dicrateria inornata* (84.4%), *I. galbana* (83.9%), *C. calcitrans* (54.2%), *T. gracilis* (48.6%) and *D. salina*

(43.2%). The above clearance rate, when fed alone, is also reflected in larvae when fed in combination, as it depended on low cell size food particles.

The clearance rate in clam spat, when fed with different diets in order of merit are 87, 86, 84, 60, 54, and 51% in *N. salina, I. galbana, D. inornata, T. gracilis, C. calcitrans* and *D. salina* respectively. The clearance rate of algal cells, when fed in combination of two algal diets showed similar trend. This also confirms, that low cell size, particle preference of larvae has reflected in the clearance rate. On statistical analysis, there is no significant difference, among various algal diets, when fed in combination as well as alone ($P \leq 0.01$). During feeding in early phase, the larvae preferred low cell size particles, among the mixed diets and observed similar clearance rate as when fed alone.

In respect to all the observations made in the present study along with other similar works in other bivalve species, the filtration and clearance rate as a function, confirms that low cell size food particle *Nannochloropsis salina* (2-3 µm) at a cell density of 5×10^3 cell/ml is ideal for larval rearing of *P. malabarica* in early stage. Also, recommend to feed, clam larvae with *N. salina* along with a flagellate, *I. galbana* during umbo, late umbo and settled spat.

Chapter 4

4. Biochemical composition of micro algae and larvae

4. 1. Introduction

Successful growth and development of cultured species depends to a large extent on the nature and biochemical content in the feed provided. These constituents are used in anabolic, metabolic processes and in creation of energy to power these processes. Physical properties of a feed, depend on the constituents, can also affect the value of food to an organism. High food value in various diets is more likely to contain the diversity of biochemical composition to satisfy optimal nutritional requirements for growth.

The metamorphosis in marine invertebrates, including bivalves, depends on the energy reserves stored up during two stages of development. The first corresponds to embryonic development, is mainly depend on endogenous reserves that parents provide in the eggs (Bayne, 1973). The second stage of development, in which stored reserves are essential to promote larval growth. This process takes place before setting and depends on the food value of diets, which are supplemented (Whyte *et al.*, 1989,1990). The use of endogenous reserves during metamorphosis reflects the inability of bivalve larvae, *D*-larvae to capture food particles during early period. During transition from larval to adult shell, the composition of shell is supposed to bind together in an organic matrix, which consists mainly of protein (Wilbur and Saleuddin, 1983). As the shell growth is continuous, the growth rate always varies according to

developmental stages of larvae (Whyte *et al.*, 1992), which directly depends on diet consumed.

Live micro algae are the main source of nutrition for filter feeding organisms (Walne, 1974; Laing and Millican, 1986). The protein component of aquaculture diets is the single most important dietary nutrients. Webb and Chu (1983) reviewed the role of chemical constituents in phytoplankton as feed for oyster larvae and spat. Kaladharan *et al.* (1999) studied the biochemical composition of six micro algal species in laboratory conditions. Algal carbohydrates were considered to be nutritionally insignificant, but proteins at high levels, when composed of the same proportion of lipids as the oyster tissue, provided high food values to algal diets. Utting (1986) has shown that settlement of *Crassostrea gigas* larvae and growth of spat were enhanced by algal diets of low protein. In many bivalve feeding experiments, larval or spat response to micro algal diets has been measured as a function of shell growth or dry weight changes rather than detailed assessment of nutritional condition of the organisms (Epifanio *et al.*, 1981; Laing *et al.*, 1987)

It is stated that results obtained in laboratory experiments using pure algal diets should not be compared with feeding response of bivalves in their natural environment, where seston is extremely heterogeneous. On the other hand, pure micro algal diets are used as algal food in bivalve hatcheries and therefore energy balance obtained under such

environmental conditions is interesting from the point of view of bivalve culture.

4. 2. Material and Methods

4. 2. 1. Biochemical composition of micro algae

The micro algae used in the present experiment were namely, *Isochrysis galbana, Chaetoceros calcitrans, Tetraselmis gracilis, Nannochloropsis salina, Dunaliella salina* and *Dicrateria sp.*. The micro algae for the biochemical analysis was taken from the out door culture prepared for feeding experiments. All the six species were harvested during exponential phase for analysis of carbohydrate, protein, lipid and ash.

The micro algal species were grown in filtered seawater (Salinity, 28-30 ppt) enriched with Conway medium (Walne, 1970) with additional silicate only for *Chaetoceros calcitrans.* Cultures were maintained at 28.6 ± 1.8°C under solar light and continuous aeration. Micro algae were harvested during the exponential phase to feed the clam larvae. Sub sample from each species culture was filtered through pre-weighed, pre combusted filter papers (Whatman GF/C, 4mm). The filters were washed with 0.5 M ammonium formate (30ml) to remove excess salt traces and dried at 100°C for 24 hrs to volatilise the ammonium formate (Epifanio, 1979).

Protein

Filters were homogenized with 4.5 ml of 10% trichloroacetic acid. The extracted protein from samples was determined using the method of Lowry *et al.* (1951).

Carbohydrate

Filters of each species were placed in Mc Carteny bottles (10 ml) together with 4.0 ml of 0.5 M sulphuric acid. The samples were heated at 100°C for 4 hours, rapidly cooled to room temperature and centrifuged at 2000 rpm for 5 minutes. Total carbohydrate in the supernatant was determined, according to Dubios *et al.* (1956) in a spectrophotometer of wavelength 480 nm.

Lipid

The filters were extracted with chloroform-methanol-water (1:2:1) mixture. The resultant supernatant from sample were combined and separated into chloroform and aqueous methanol layers by the addition of chloroform: methanol: water of 1:1:1 (Bligh & Dyer, 1959). These layers were concentrated under vacuum and weighed to determine total lipid.

4. 2. 2. Biochemical analysis of clam larvae

As the sufficient quantity of larvae necessary for analysis, the fertilized eggs were transferred and reared in separate tanks. Larvae were fed with all the above-mentioned micro algal species singly. Samples for biochemical analysis were removed at '*D*'-shape, umbo, veliger and spat

by using appropriate sieves. All the larval samples washed with 0.9% ammonium formate, isotonic with seawater, to remove traces of salt.

Ash

Inorganic matter was estimated gravimetrically after ashing 20 mg larval sample in a muzzle furnace at 70-80°C for 24 hrs.

Protein

Larval sub samples were extracted and hydrolysed using 0.5 N Sodium hydroxide for 24 hrs at 30°C. Protein was assayed as described by Lowry *et al.* (1951). Absorbance was read at 530 nm in a UV/VIS spectrophotometer.

Carbohydrate

Larval homogenate for total carbohydrate was dissolved in 15% trichloroacetic acid. Method specified by Holland and Gabbott (1971) was used with glucose as standard. Absorbance was read at 420 nm in a spectrophotometer.

Lipid

Samples were extracted in chloroform: methanol (1:2) mixture. After standing at 4°C for 10 minutes, lipid dissolved in chloroform was extracted by centrifuging at 6000 rpm. Aliquots of the sample extract were quantitatively estimated by the method of Marsh and Weinstain (1966) using tripalmitin as standard. After drying of sample at 37°C, Sulphuric acid was added and heated for 15 minutes at 200°C. Samples were spectrometrically read at 375nm.

4. 3. Results

4. 3. 1. Biochemical composition of micro algae

The gross biochemical composition of the micro algae used in the present study was determined from replicate cultures. The compositional data presented in Table 1 shows the average values for these pooled analyses on dry matter basis. The supplementary micro algae assessed differ in their proximate composition (Table 3. 1.)

Table 3. 1. Mean biochemical composition of micro algae tested in experiments

Micro algae tested	Proximate composition (%)		
	Protein	Carbohydrate	Lipid
Nanochloropsis salina	53 ± 4	36 ± 4	22 ± 4
Chaetoceros calcitrans	41 ± 5	21 ± 3	15 ± 2
Tetraselmis gracilis	37 ± 3	29 ± 2	16 ± 4
Isochrysis galbana	38 ± 3	30 ± 3	24 ± 5
Dunaliella salina	36 ± 8	28 ± 5	23 ± 2
Dicrateria inornata	30 ± 4	26 ± 5	21 ± 3

In micro algae, the protein level is high among the proximate principles. This is highly reflected in the larval growth and settlement in the present study. The protein content in the micro algae tested in order of merit is written as *N. salina* > *C. calcitrans* > *I. galbana* > *T. gracilis* > *D. salina* > *D. inornata*.

The mean carbohydrate level in algal species *N. salina*, *I. galbana*, *C. calcitrans*, *T. gracilis*, *D. salina* and *D. inornata* was 36 %, 30 %, 21 %,

29 %, 28 % and 26 % respectively the highest lipid level was observed in *I. galbana* (24 %) and lowest in *C. calcitrans* (15 %).

On statistical analysis, only in case of protein content highly significant among the proximate composition is observed ($P< 0.05$).

4. 3. 2. Biochemical composition of clam larvae when fed with different micro algae

The proximate composition of larvae fed with different micro algae was determined during the stage *D* larvae, umbo and settled spat. The samples were pooled together irrespective of size difference during each stage for evaluation. However, the samples were taken as per requirement for the analysis.

Larvae fed with monoalgal diet

Protein

Initial protein concentration of *D* larvae was uniform irrespective of algal species (84 ± 1 µg ind^{-1}). Maximum protein level was observed in the umbo stage fed with *N. salina* (76 ± 1 µg ind^{-1}) and larvae fed with *D. salina* have relatively low protein. (41 ± 3 µg ind^{-1}). The highest (182 ± 0 µg ind^{-1}) and lowest (129 ± 2 µg ind^{-1}) protein level observed in settled spat fed with above-mentioned algal species. The protein concentration of 3 larval stages on development, when fed with different micro algae is represented in Figure 4. 1.

Carbohydrate

The highest carbohydrate concentration was recorded in larvae fed with *N. salina* (15.5 ± 1 µg ind^{-1}) and lowest in *I. galbana* (11.4 ± 1 µg ind^{-1}), after spat settlement. The observed high and low concentration in umbo stage is in larvae fed with *T.gracilis* (10.5 ± 2 µg ind^{-1}) and *D. salina* (7.3 ± 1 µg ind^{-1}) respectively. The "*D*' larvae did not show no much variation on various algal species. The carbohydrate level of clam larvae during development is represented in Figure 4. 2.

Lipid

There was not much variation in lipid level in '*D* ' larvae and umbo irrespective of the algal species fed, during the present study. However, a low lipid level was observed in *D. inornata* (14.5 µg ind^{-1}). The highest and lowest lipid concentration of settled spat was observed in larvae fed with *T. gracilis* and *D. salina* respectively. The lipid concentration of larvae on development, when fed with six micro algal species tested is represented in Figure 4. 3.

Abbreviations for Fig. 4.1 – 4. 3.

I.g = Isochrysis galbana, N.s = Nanochloropsis salina, C.c = Chaetoceros calcitrans, D.i = Dicrateria inornata, T.g = Tetraselmis gracilis and *D.s = Dunaliella salina.*

Biochemical composition of clam larvae on development

Fig. 4. 1. Protein

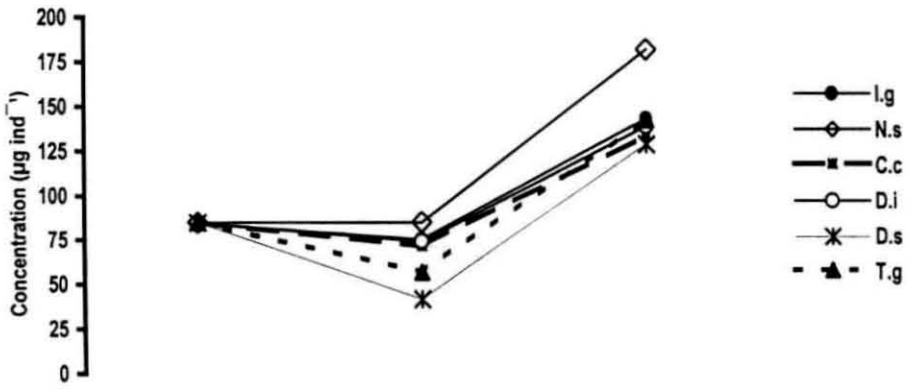


Fig. 4. 2. Carbohydrate

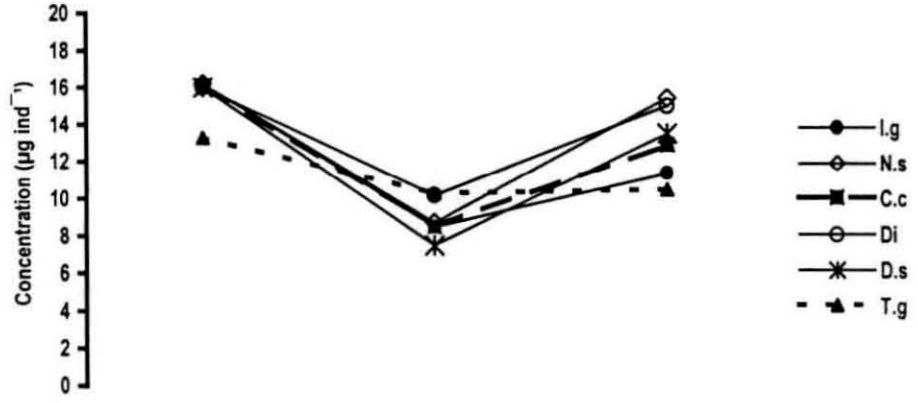
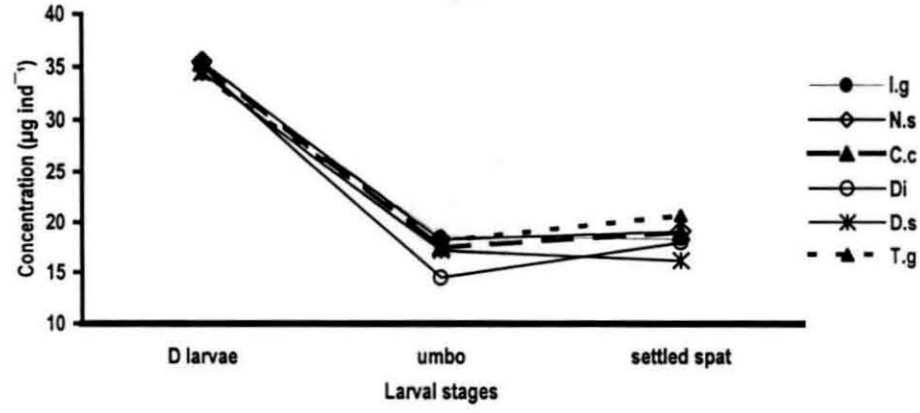


Fig. 4. 3. Lipid



Larvae fed with Mixture of two algal species

The survival of larvae on various combined algal species was low. Hence, only which gave a moderate survival in each stage on observations during the study, were taken for the analysis of biochemical composition. The following algal combinations were tested and gave sufficient quantity for the analysis and other combination was not considered for the evaluation. The combinations are *I.g + C.c, N.s + I.g, N.s + T.g, N.s + D.i, D.s + T.g, C.c + T.g, T.g + D.i, N.s + D.s* and *N.s + C.c.* (abbreviations as described in above section).

Protein

There is no much dietary variation in '*D* ' larvae, when fed with various algal combinations (84.9 ± 1.1 µg ind^{-1}). In the umbo stage, the highest protein level is represent by the combination of *I. galbana* and *C. calcitrans* (183 ± 1µg ind^{-1}) and lowest in *I. galbana* and *D. salina* (69.3 ± 0 µg ind^{-1}). However, the high survival was observed in the algal combination *N. salina* with *I. galbana* with estimated protein concentration of 177 ± 1.3 µg ind^{-1}. The protein level of clam larvae on development when fed with mixed algal species is represented in Figure 4. 4.

Carbohydrate

Only three algal combinations were significantly showed high carbohydrate level during larval development among all the algal combinations tested. They are combination of *N. salina* and *I. galbana, N. salina* and *D. inornata* and *I. galbana* with *D. inornata.* However, there is no significance among these three algal combinations ($P > 0.05$). The

carbohydrate level of clam larvae on development when fed with mixed algal species is represented in Figure 4. 5.

Lipid

Lipid level of *'D'* larvae, umbo and spat settled when fed with mixed algal combinations is represented in Figure 4. 6. High lipid concentration was observed in *'D'* larvae (35.7 ± 0.9 µg ind^{-1}) and as on development lipid level was decreased in umbo and later enhanced a gain considerably. The highest level was observed in larvae fed with combination of *N. salina* with *I. galbana* (18.7 ± 1 µg ind^{-1}) and lowest in combination of *D. salina* and T. *gracilis* (10.4 ± 1.2 µg ind^{-1}). The lipid concentration on other algae with *N. salina* was observed higher among all the combinations tested. The mean lipid level of *D. salina, T. gracilis* and *D. inornata* with *N. salina* was 18.2, 17.9 and 17.4 µg ind^{-1} respectively.

4. 3. 3. Biochemical composition of spat

The proximate composition of juveniles reared in the present study shows no much variation among various algal diets. However, the algal combinations gave better result than mono algal diet. The observed dietary protein level was high among the biochemical composition. The compositional value for mono algal diet and mixed diets are represented in Table 4. 2. The results on statistical analysis show significant within the mixed diets, composed of *N. salina* with *Tetraselmis gracilis, Chaetoceros calcitrans, D. salina* and *I. galbana* in which, the last mentioned species combination is prominent ($P< 0.05$).

Biochemical composition of clam larvae on development

Fig. 4. 4. Protein

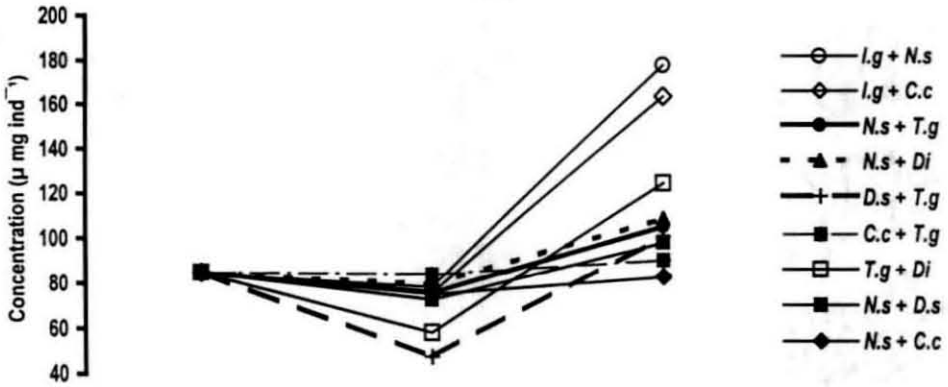


Fig. 4. 5. Carbohydrate

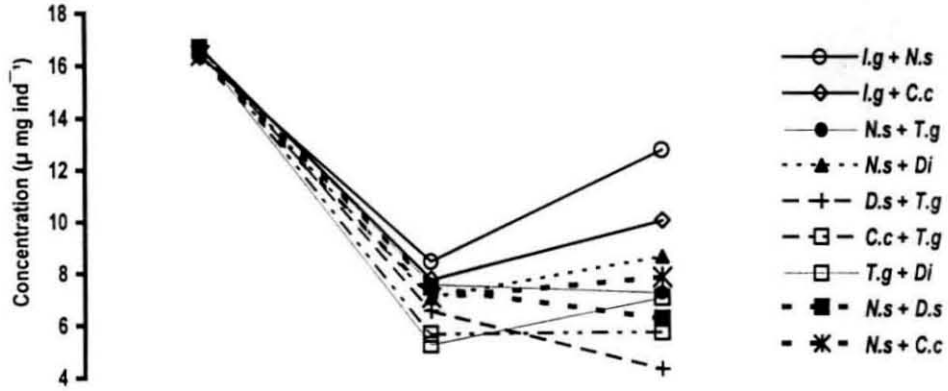


Fig. 4. 6. Lipid

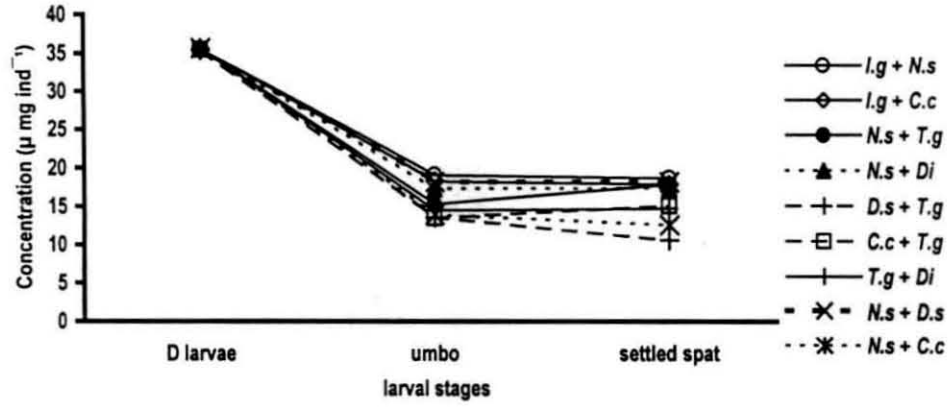


Table 4. 2. Biochemical composition of spat

Micro algae tested	Biochemical composition (µg ind^{-1})					
	Protein		Carbohydrate		Lipid	
	Initial	Final	Initial	Final	Initial	Final
Mono diet						
N. s	177.5	294.7	14.9	20.2	15.9	22.9
I. g	143.8	215.6	11.0	16.7	11.5	18.2
C. c	134.1	208.3	12.6	17.2	18.7	23.1
D. i	139.4	236.7	14.7	19.0	16.4	24.3
T. g	142.1	213.8	14.5	19.1	19.8	27.1
D. s	139.4	211.4	13.9	19.0	18.1	25.7
Mixed diet						
N. s + I. g	182.3	312.2	12.5	18.7	18.0	31.0
N. s + C. c	81.8	197.4	8.1	18.6	12.5	23.6
N. s + D. s	98.2	198.0	6.1	18.4	18.0	31.4
N. s + T. g	105.0	207.1	7.2	14.7	18.0	29.1
N. s + D. i	108.0	197.3	8.3	17.8	16.8	30.3
I. g + C. c	163.2	243.4	10.1	16.2	17.7	27.1
I. g + D. i	145.2	216.0	10.2	14.2	15.0	23.6
I. g + D. s	97.6	167.9	10.2	15.4	14.8	19.2
I. g + T. g	68.9	158.2	8.0	13.6	11.2	20.3
C. c + D. s	80.8	150.9	5.9	8.4	12.5	17.5
C. c + T. g	80.8	153.6	7.0	11.2	12.2	19.4
D. s + T.g	100.5	190.4	8.7	15.8	15.9	24.3
D. s + D. i	94.0	177.0	6.5	14.1	12.5	18.4
T. g + D.i	124.0	204.6	6.9	9.3	14.1	25.3

4. 4. Discussion

Micro algae are used extensively in bivalve culture as food for larvae and juveniles. The biochemical composition of micro algae for its nutritional quality has been tested, but not all species are equally successful in supporting optimum nutritional requirements for larval growth and spat production in many species (Davis and Guillard, 1958; Walne, 1970; Epifanio *et al.*, 1981; Enright *et al.*, 1986; Labarta *et al.*, 1999; Laing, 1993 and Albentosa *et al.*, 2000). The comparison of biochemical composition of algal diets and resultant clam larvae proved that dietary protein played a critical role in nutritional conditioning of larvae.

The settlement and metamorphosis of the larvae are among the most important periods in life cycle of bivalves, during which dramatic changes in morphology, physiology and habitat occur (Chia, 1989). These changes are essential for the transition from a larval to an adult phase (Bayne, 1971), but high mortality has been recorded during these stages, especially at metamorphosis. All the transition changes are accompanied by an increase in development of organ systems. This transition phase, from pelagic to benthic is generally considered as an energy consuming, critical stage in the life cycle of many species (Rodriguez *et al.*, 1990). One cause of high mortality is an inadequate supply of energy in the larva before metamorphosis (Mann, 1988) and there is a minimum energy requirement for the successful completion of metamorphosis (Gallager and Mann, 1986). Thus the energy consumed by larva was established and that lipids represent major energy source in veligers (Holland, 1978;

Mann and Gallager, 1985; Ferreiro *et al.*, 1990). Bartlett (1979) found that *Crassostrea gigas* accumulates more energy in the form of proteins than lipids, which forms the basic energy reserve during metamorphosis in *Ostrea edulis* with 69.3% of the total energy required. Some others indicated that lipids are the principal energy substrate utilised during the process of settlement and metamorphosis including early juvenile stages. But such reports might be the result of different experimental conditions such as nutrition, antibiotics, temperature, larval density etc. In general, micro algal species used as food for larvae and adults in laboratory vary considerably in nutritional content. There are changes in the chemical composition of the algal culture at different phases of growth. Studies on clam *Mercenaria mercenaria* (L.) have shown that the type of micro algae used as food, the algal phase in which algae harvested and nutrient conditions which influence the biochemical composition of algae and thus the rate of growth of the bivalve (Wikfors *et al*, 1992).

The cell concentrations of all the biochemical constituents vary with different culture conditions (Wikfors *et al.*, 1984; Ben-Amotz *et.al.*, 1985). To avoid such problems, all micro algae were grown under similar conditions and harvested at the same growth phase, to allow comparison of composition between species tested in the present study.

With respect to proximate composition total protein, lipid and carbohydrate in micro algae can vary substantially with the species and culture conditions (Parsons *et al.*, 1961; Dortch, 1982; Wikfors *et al.*,

1984; Ben-Amotz *et al.*, 1985; Fabregas *et al.*, 1986; Moal *et. al.*, 1987; Renaud *et al.*, 1991). The levels of these major biochemical fractions may be related to the nutritional quality of micro algae. Hence, in the present study, the proximate composition analysis of the algae tested could make it possible to evaluate specific optimum nutritional requirements for larval rearing and spat production of the yellow clam, *P. malabarica*. The concentrations of protein, carbohydrate and lipid in the six species of micro algae are compared in Table 4. 1. In the present study, protein was always the major organic constituent followed by lipid and then carbohydrate. More over, different methods of analysis can also complicate comparisons of biochemical data. This is particularly in case of protein analysis, as Kjeldahl method (Parsons *et al.*, 1961; Whyte, 1987), Lowry method (Moal *et al.*, 1987; Fernandez-Reiriz *et.al.*, 1989) or Comassie blue method (Fabregas *et al.*, 1986; Bartlett, 1979). Different protein values are obtained from same sample by these different methods (Lohrenz and Taylor, 1987: and Clayton *et al.*, 1988). In the present study, the protein value obtained by Lowry method shows that *N. salina* was highly proteinacious and gave early settlement of spat and maximum survival. The algal combination of *Nanochloropsis salina* with *I. galbana* also proved a similar trend.

The proximate composition of micro algae is comparable to similar works done, for evaluation of suitable micro algal feed, based on biochemical analysis (Walne, 1970; Chu *et al.*, 1982; Fabergas *et al.*, 1985; Volkman *et al.*, 1989; Assaf Sukenik and Rachel Wahnon, 1991;

Brown *et al.,* 1989; Sukenik *et al.,* 1993; Camacho, 1998; Malcom *et al.,* 1999; Kaladharan *et al.,* 1999). The micro algae *Nanochloropsis salina*, used in the present study, contained twice the proportion of carbohydrate (52%) compared to all other species tested. This was supported by another species of *Nanochloropsis* (*N. atomus*) (Brown *et al.,* 1989,1991), while comparing with oyster *C. gigas* larvae to assess the protein quality of micro algae. Thus in the present study, based on protein quality, the micro algal species is written in the order of merit as *Nanochloropsis salina* > *Chaetoceros calcitrans* > *Isochrysis galbana* > *Tetraselmis gracilis* > *Dunaliella salina* > *Dicrateria inornata.*

With respect to biochemical charges on clam larvae, the micro algal species *N. salina, D. inornata* and *I. galbana* have promoted excellent growth, when used as feed during early larval development ie., '*D*' larvae to umbo. The high protein and carbohydrate level in *N. salina* gave maximum survival and spat production during these experiments. It is also observed that algal combination with *I. galbana* gave maximum spat production and all the major organic components are comparatively high among all other algal combinations. The result of such an earlier study in *N. atomus*, which is deficient in PUFA, when fed in conjunction with algae rich in 20:5 ω 3 and 22: 6ω3, gave good result in nutritional conditioning of scallop larvae (Volkman *et al.,* 1989).

Regarding the carbohydrate and lipid values obtained in the present study is not very significant for larval growth and spat production.

However, it showed a marked difference in seed/ juvenile culture of clam. All the algae tested in the experiments gained maximum growth and energy in terms of carbohydrate and lipid (Table 4. 2). The studies on adult, clam, *Villorita cyprionoides* (Chinnama George and Gopakumar, 1995; Gireesh *et al.,* 2001) and in adult *Paphia malabarica* (Appukuttan an Aravindan, 1995) showed that protein and fat content are equal in composition. Earlier works on bivalve spat in temperate species confirmed that micro algal species provides carbohydrate and protein content more than total lipid (Millican *et al.*, 1986 and Enright *et al.*, 1986). Albentosa *et al.,* (1999) found that mixture of *I. galbana* and *T. gracilis* influenced the growth and development of clam, *Venerupis* spat and confirmed with the protein index value of the seed. Many of the works done in oysters and mussels, also confirms that the dietary protein is the major nutrient source of larval growth and settlement.

In conclusion, micro alga *Nanochloropsis salina* proved to be a good alternative feed to *Isochrysis galbana* in larval rearing and spat production, while evaluating the effect of organic composition of micro algae tested in the present study. The combination of the above mentioned species also showed better results. However, in seed culture, all the micro algal combination with *N. salina* shown good results, especially with *T. gracilis, C. calcitrans* and *D. salina.*

Chapter 5

5. Effect of Salinity and pH on algal nutrition for larval development and spat production of *Paphia malabarica*

5. 1. Introduction

Environmental conditions, besides algal diets, influence the growth and reproduction of bivalves. A wide range of fluctuations in the environmental conditions will also affect the survival and development of larvae. Among the various environmental factors, salinity, temperature and pH were the main factors affecting development of bivalve larvae (Bayne, 1976). Typically, temperature influences survival and growth and salinity affects more than growth. Bayne (1976) studied the effect of temperature and salinity on bivalve larvae and settlement in natural conditions. The spawning season and release and development of larvae are usually synchronized with favourable environmental conditions. The survival and behaviour of oysters in lower salinities have been studied by Loosanoff (1948, 1950, and 1952), Ingle and Dawson (1950) and David (2000). The combined effect of algal feed and temperature on veliger larvae of gastropod was carried out by Davis (2000) and Aspari and Anshary (1997) on effect of temperature and salinity on clam, *Tridacna gigas*. Very few works have been done on, survival and development of bivalves from Indian waters, especially in clams, in case of, salinity tolerance and pH levels. In India, behaviour of clams *Meretrix meretrix* and *Katylesia opima* in low water salinities (Ranade and Kulkarni, 1974), effect of temperature and salinity on growth and feeding rate of *Villorita*

cyprinoides (Preetha and Nair, 1993) and on salinity tolerance in the adult yellow clam, *Paphia malabarica* were studied (Rammohan and Velayudhan, 1998). The effect of various chemicals presented in seawater also influences the larval survival and spat production of the mussel *Perna indica* (Manoj, 2000). However, no works have been carried out in clam larvae during its development. As clams being brackish water forms and more prone to environmental fluctuations, during spawning season and monsoon, it is necessary to identify the level of salinity tolerance as well as pH level for their culture and production of spat and seed culture.

Generally all the studies related to standardisation of hatchery protocol have been carried out as univariate experiments (Bayne, 1976; Loosanoff, 1950) with a single factor at a time or as a multivariate experiments (David, 2000) with two or more factors at a time. All the above results have been used to identify ideal rearing conditions.

Considering the importance from the management point of view, the effect of salinity and pH on larval rearing of the clam, *Paphia* malabarica were studied in detail during the present work. The present study was carried out in a single factor form and aims at studying the effect of the same individually on clam larval growth and setting. This will also help to identify the effect of salinity and pH on sites during spawning season as well as to standardize the hatchery technology.

5. 2. Material and Methods

The effect of salinity and pH on clam larval growth and setting were studied individually and with different broods of larvae. *Nanochloropsis salina* at a density of 5 X 10^3 cells ml^{-1} was used as food. The control temperature, salinity and pH were 27.5 ± 1.0 °C, 32.5 ± 0.7 $^{o}/_{oo}$ and 8.15 ± 0.20. The general rearing conditions were maintained as described in chapter 2.

5. 2. 1. Effect of salinity on larval development

Experiments were conducted to study the effect of salinity on clam larval rearing when, seawater of the required salinities was prepared prior to water exchange. Filtered seawater was taken in a vessel and diluted with filtered freshwater until the desired salinity was obtained. The derived formulae to obtain required salinity was,

$$\text{Desired salinity (ml)} = \frac{\text{Volume of desired salinity}}{\text{Volume of known salinity}} \times 100$$

Salinity was monitored using a salinometer. Larvae were acclimatised to the lower salinities gradually by a decrease of 5 $^{o}/_{oo}$/day. Larvae were exposed to salinity ranges 10, 15, 20, 25, 30, 35 and 40 $^{o}/_{oo}$ with 33 $^{o}/_{oo}$ as control (Plate 5 b).

5. 2. 2. Effect of pH on larval development

Larvae were exposed to five pH levels of 7.5, 8.0, 8.5, 9.0 and mean control pH 8.12 (8.10-8.14). For raising the pH level of the medium to above that of the ambient pH of 8.12, Tris buffer of pH 7.0-9.0 was used, while bringing pH of the medium to below that of ambient pH,

solution of citric acid was used. Seawater of required pH level was prepared just prior to water exchange. Filtered seawater was taken in a vessel and either Tris buffer or citric acid solution was added and mixed thoroughly until the pH meter reading was at the desired level. Later samples were monitored (Plate 5 a).

5. 2. 3. Effect of salinity on spat

Experiments were conducted to study the effect of salinity on spat (mean size of 1 ± 1.0 mm). Spat about 50 numbers, were reared in 5-litre container for 30 days. For experiments, seawater of desired salinity was prepared prior to water exchange as described in previous section.

5. 2. 4. Effect of pH on spat

Spat about 50 numbers, with a mean length of 1 mm were reared for the 30 days experiment. The average ambient temperature, salinity and pH during this experiment were 28.6 – 29.4°C, 32.1-33.5 $^{o}/_{oo}$ and 8.3-8.5. The juveniles were reared at four pH levels of 7.5, 8.0, 8.5 and 9.0 and ambient pH of 8.15 (8.12-8.18) for 30 days. The method of preparing seawater of desired pH levels was mentioned in the preceding experiment.

Growth (anterior posterior measurement), growth rate, survival of umbo and spat, rate of survival and number of setting days were taken as criteria to evaluate the best and optimum salinity and pH requirement during larval rearing of *Paphia malabarica*.

Plate 5

a) **Experimental set up: To study the salinity tolerance of spat**

b)) **Experimental set up: To study the pH level on larval rearing.**

Plate 5

a

b

Growth (mean anterior posterior measurement), growth rate and rate of survival were taken as criteria in case of juvenile/ spat culture of this clam.

5. 3. Results

5. 3. 1. Effect of salinity on larval development

Growth

The larval development in various salinities till settlement on 15th day is given in Figure 5.1. Larvae reared in various salinities showed a uniform growth at earlier stage of metamorphosis. The mean size of early umbo on 6th day was 118.4, 115, 120, 121 and 140.5 µm in 10, 15, 20, 25 and 30 $^{o}/_{oo}$ salinities respectively. The mean size of umbo in ambient salinity (33%) was 138 µm, with not much variation from the above ranges. However, a marked difference in anterior posterior measurement was observed from pediveliger till settlement. The mean size of spat settled in above-mentioned salinity were 125, 125, 128, 136 and 182 µm respectively with a mean size of 182.3 µm in 33 $^{o}/_{oo}$.

The mean size of spat settled was significantly ($P<.01$) high in salinity 30-33 $^{o}/_{oo}$ than the range of 10-25 $^{o}/_{oo}$. The analysis of covariance showed no significance between salinity 30 and 33 $^{o}/_{oo}$ ($P \geq 0.005$). The larvae did not survive beyond day 5 in higher salnities of 35 and 40 $^{o}/_{oo}$. Table 5.1 shows summary of the mean larval anterior posterior measurement in different stages.

Fig. 5. 1. Larval growth when reared at different salinities till settlement

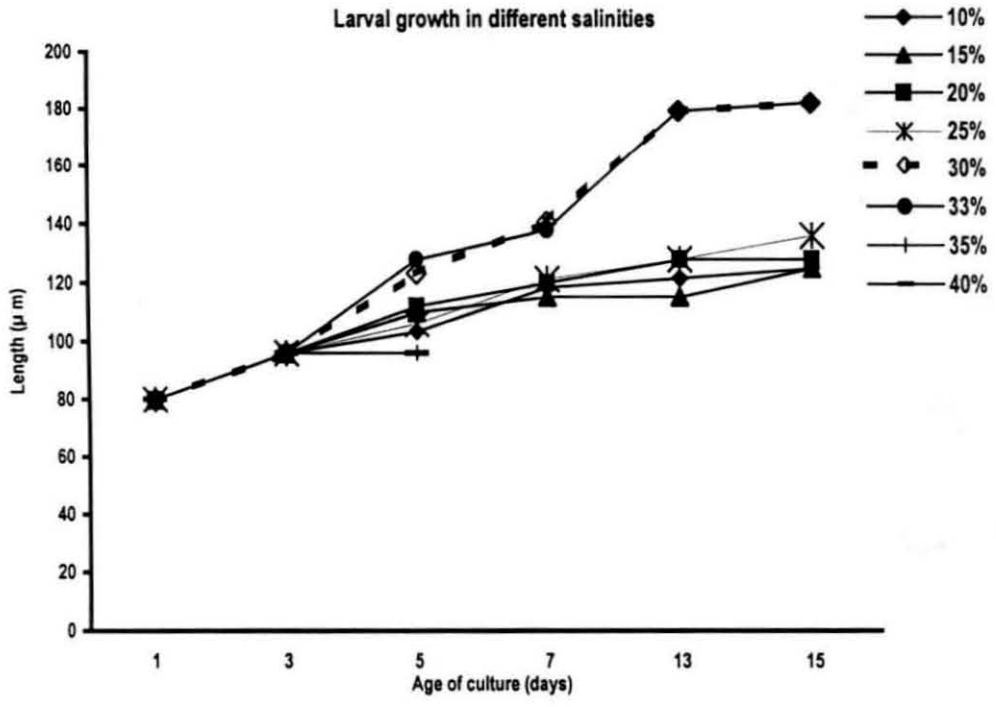


Table 5. 1. Mean size of larvae at different salinities during development

Salinity (°/₀₀)→ No. of days ↓	Mean size of larvae (μm)							
	10	15	20	25	30	35	40	33 (control Salinity)
2	80 ± 0	80 ± 0	80 ± 0	80 ± 0	80 ± 0	80 ± 0	80 ± 0	80 ± 0
3	96 ± 2	96 ± 0	96 ± 0	96 ± 0	96 ± 0	96 ± 0	96 ± 0	96 ± 0
5	103 ± 3	110 ± 1	112 ± 2	106 ± 2	123 ± 2	96 ± 0	96 ± 0	128 ± 2
7	118 ± 2	115 ± 2	120 ± 3	121 ± 3	140 ± 3	-	-	138 ± 3
11	121 ± 2	115 ± 3	128 ± 3	128 ± 2	179 ± 2	-	-	179 ± 2
15	125 ± 2	125 ± 3	170 ± 4	169 ± 3	182 ± 3	-	-	182 ± 2

The length size frequency of spat on 15th day varied in different salinities (Fig. 5. 2 and 5. 3). The 90 % larval size frequency 160-180 μm,

are observed in larvae, reared at salinity 20 °/₀₀, with a mean size 170 µm on 15th day. The salinity 30 °/₀₀ range showed a high size frequency (180-200 µm) with 70 % spat settled in this range. The same was observed in ambient salinity 33% with 90 % of spat in the size frequency of 180-200 µm. No larvae reared in other salinities showed such a size frequency pattern during this experiment. In salinities 10 °/₀₀ and 15 °/₀₀, 70 % of spat are in range of 160-180 µm, where it is 90%, in the case of salinity 20 °/₀₀.

Growth rate

The highest growth rate of 6.3 µm/day till umbo stage was observed in salinity 30 (±1) °/₀₀, while it was 5.4, 5.1 and 5.1 µm /day in salinity 10, 15 and 20 °/₀₀. In salinity 25 °/₀₀ the growth rate observed was 5.9 µm/day. The variation in growth rate in salinity 30 °/₀₀ and in control salinity 33 °/₀₀ showed high significance among other treatments. The growth rate that observed in salinities 10, 15, 20, 25, 30 and control salinity 33 °/₀₀ are 0.732, 0.707, 0.707, 0.707, 0.770 and 0.857 log µm/day respectively.

The growth rate from umbo stage upto settlement showed a significant increase while the salinity increased. The growth rate was 0.8, 1.3, 1.0 and 1.8 µm/day in salinity 10, 15, 20 and 25 °/₀₀ respectively. However, a high growth rate of 5.1 was observed in the same period in salinity 30 and 33 °/₀₀. This clearly indicates that clam larvae should be reared in the salinity range 30-33 °/₀₀. The growth rate during the same period showed significance.

Fig. 5. 2. Size Frequency percentage of clam larvae reared and settled in different salinities on day 15

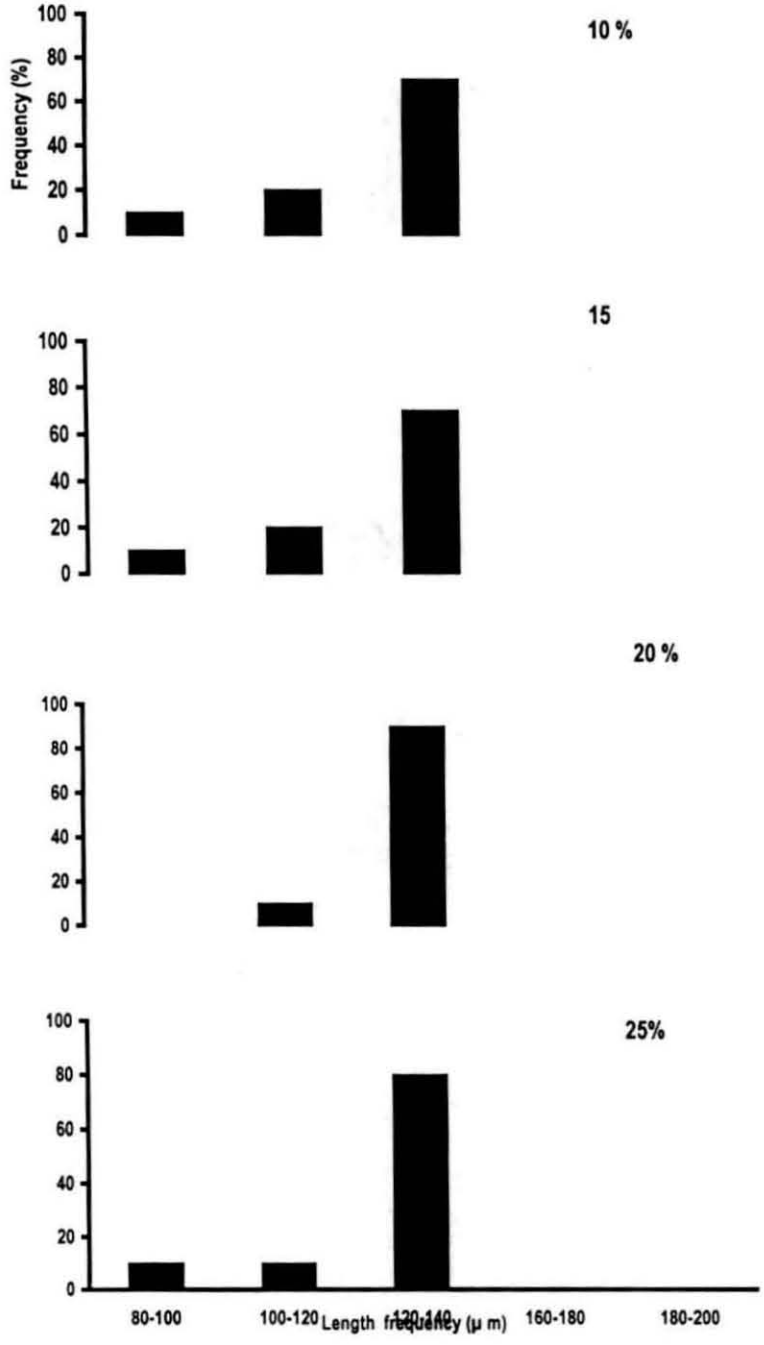
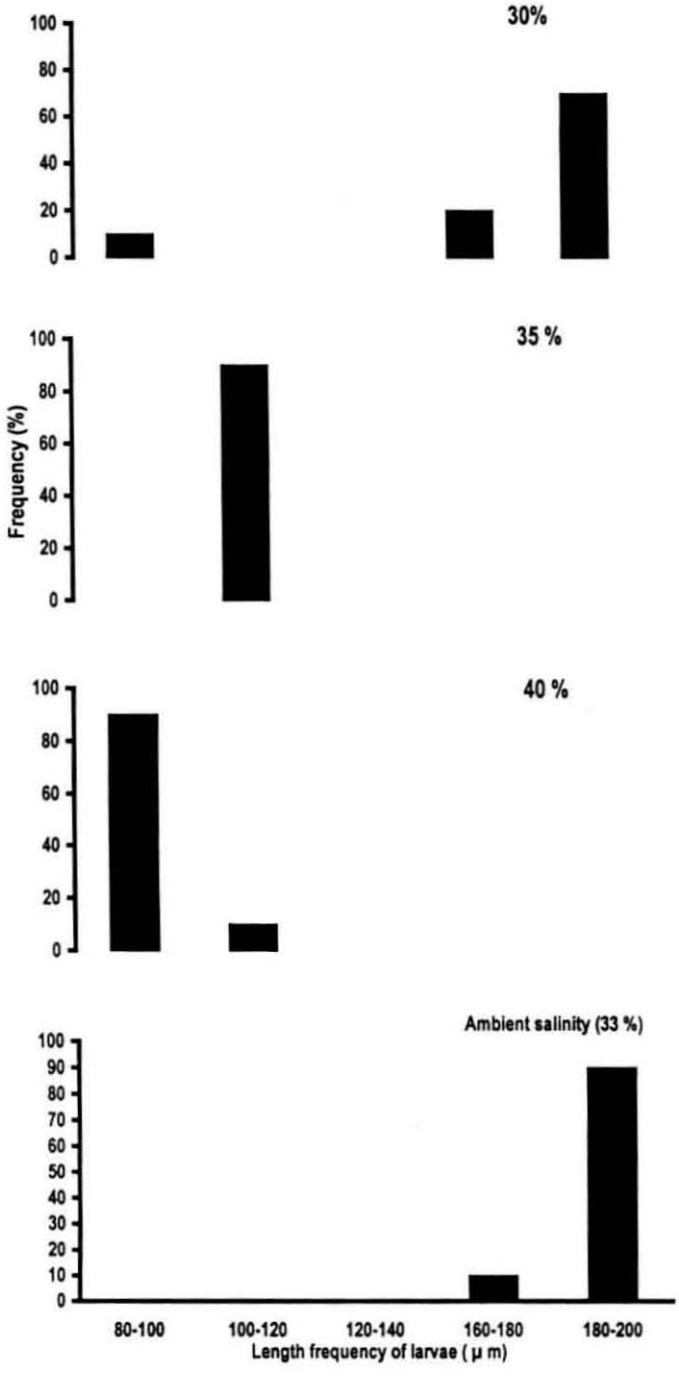


Fig. 5. 3. Size Frequency percentage of clam larvae reared and settled in different salinities on day 15



between treatment (P≥ 0.05). The observed growth regression rate was 0.009, 0.113, 0, 0.255, 0.707 and 0.747 log µm/day in salnitiies 10, 15, 20, 25, 30 and 33 $^{o}/_{oo}$.

The overall growth rate of spat settled in above salinities on 15th day was 3.0,3.2, 3.7, 6.8 and 6.81 µm/day. The overall growth rate (Table 5. 2) showed significant difference between treatments (P≥ 0.05) and the values are summarised in Table 5. 3.

Table 5. 2. Growth rate of clam larvae reared at different salinities

	Mean Growth rate (µm/day)					
Stage	10 $^{o}/_{oo}$	15 $^{o}/_{oo}$	20 $^{o}/_{oo}$	25 $^{o}/_{oo}$	30 $^{o}/_{oo}$	33 $^{o}/_{oo}$
D – Umbo	5.4	5.1	5.1	5.9	6.3	7.2
Umbo-Spat	0.8	1.3	1.0	1.8	5.1	5.5
D - spat	3.0	3.0	3.2	3.7	6.8	7.0

Table 5. 3. ANOVA. Larvae reared at different salinities

Source	Analysis of Variance				
	Sum of Squares	df	Mean Square	F- ratio	P
Salinity	139048.96	7	19864.13	395.78	0.000
Days	251940.48	4	62985.12	1254.94	0.000

Survival

Maximum survival of 91.8 % in umbo stage was observed in control salinity 33 ± 1 $^{o}/_{oo}$. The second highest survival of 17.8 % was observed in salinity of 30 $^{o}/_{oo}$. Less than one tenth of total larvae developed into umbo stage and showed an increase in survival with

increase in salinity (Fig. 5. 4). The observed percentage of survival was 2.8, 2.9, 6.9 and 5.9 % in salinities 10, 15, 20 and 25 $^{o}/_{oo}$ respectively. No larvae survived in higher salinities of 35 and 40 $^{o}/_{oo}$.

The same pattern of survival was observed in spat settlement (Fig. 5. 5). 89.1 % of umbo in salinity 30 $^{o}/_{oo}$ is settled, while it was 93.4 % in control salinity. Half of the umbo were developed and settled in salinity 20 and 25 $^{o}/_{oo}$. An increase in settlement with salinity increase was observed upto 33 $^{o}/_{oo}$ beyond which survival reduced. The observed percentage survival of umbo till settlement was 24.5, 38.1, 50.9 and 51.8 % in salinity 10, 15, 20 and 25 $^{o}/_{oo}$ respectively (Fig. 5. 5).

The overall survival rate of spat settled is 0.7, 1.1, 3.5, 3.1, 15.4 and 85.5 % in salinities 10,15,20,25, 30 and control salinity 33 $^{o}/_{oo}$.

No. of days taken for spat settlement

Table 5. 5 summarise the days taken for settlement of larvae in various salnities. The early initial settlement on day 9 was observed in salinity 30 %o. The complete settlement was observed by 11th day. The initial settlement was high compared to final settlement in the above salinity. Spat settlement was delayed in lower salinities. The initial settlement in salinity 25 and 20 % was observed in day 11 and prolonged for another 4 days for complete settlement. This was clearly reflected in the development of larvae, growth and survival. In control salinity, the initial settlement was observed on day 10 and complete spat settled by next day.

Fig. 5. 4. Percentage of survival of umbo and spat settlement in different salinities

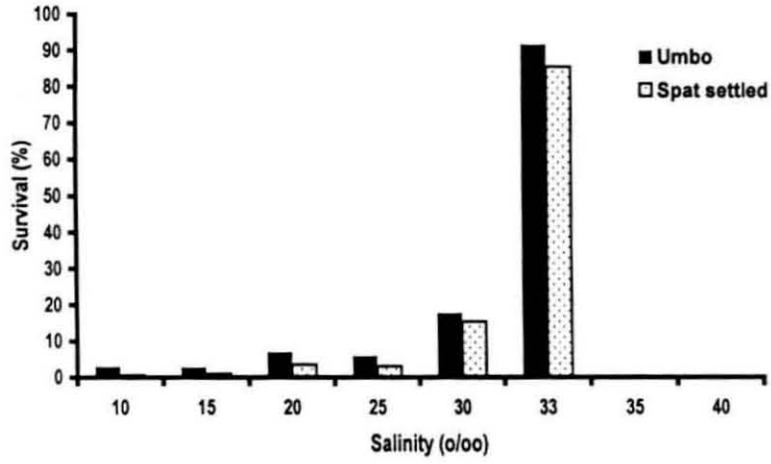
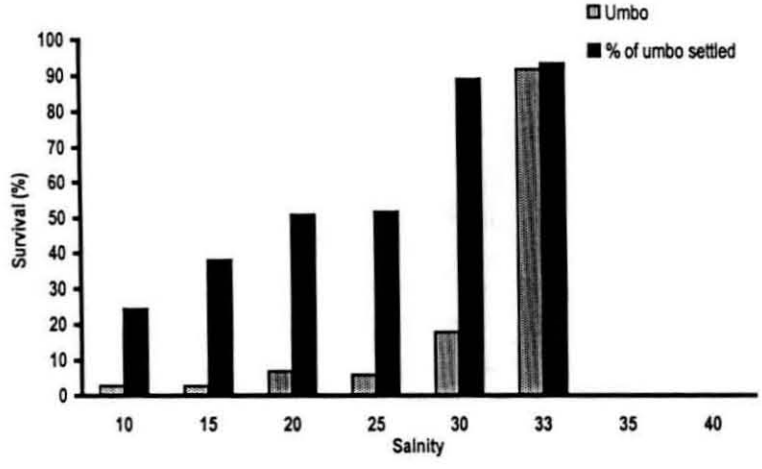


Fig. 5. 5. Percentage of Umbo settled during development in different salinities



Algal cell consumption

The consumption of algal cells was high in salinity 25 $^{o}/_{oo}$ and 30 $^{o}/_{oo}$ (Table 5. 4). The *D* larva showed low algal cell consumption in lower salinities of 10, 15 and 20 $^{o}/_{oo}$. Larvae reared in salinity 25 $^{o}/_{oo}$ in early stages of development consumed 50 % algal cells. It was observed that with the development of larvae, the consumption of algae also increases in all the salinities. The larvae reared in control salinity shows a maximum algal consumption of 93, 90 and 82 % in *D* larvae, umbo and spat respectively.

Table 5. 4. Algal cell consumption during larval rearing at different salinities

Algal cell consumption (%)								
Salinity (%) >	10	15	20	25	30	35	40	33 (Ambient salinity)
D larvae	20	24	30	50	90	-	-	93
umbo	28	28	38	70	90	-	-	90
spat settled	35	35	58	70	85	-	-	82

Table 5. 5 Effects of different salnities on clam larval growth and setting

Salinity (%)→ No. of days ↓	Larval Growth and setting of Clam larvae							
	Mean size of larvae (µm)							
	10	15	20	25	30	35	40	33 (Ambient Salinity)
2	80	80	80	80	80	80	80	80
3	96	96	96	96	96	96	96	96
5	103	110	112	106	123	96	96	128
7	118	115	120	121	140	-	-	138
11	121	115	128	128	179	-	-	179
15	125	125	128	136	182	-	-	182
Day of 1st setting	12	12	11	11	9	-	-	10
Final setting	15	15	15	15	11	-	-	11
Total No. of spat	285	275	350	310	1535	-	-	8580
Spat production (%)	2.85	2.75	3.5	3.1	15.4	-	-	85.8

5. 3. 2. Effect of pH on larval development

Growth

The larvae reared in pH level 8.0 and ambient pH 8.1 showed a maximum mean growth size of 185 and 184.9 µm respectively (Fig. 5. 6). The mean size of early umbo was observed as 139 µm in both pH levels. In a higher pH of 8.5, the observed mean size of umbo and spat was 134 and 178 µm respectively. The larvae reared at lower pH levels 7 and 7.5 and in high pH 9.0 did not develop and survive beyond *D* shape larval stage (Table 5. 6). Analysis of Covariance showed no significance among various pH treatments ($P \leq 0.05$).

The larval growth size frequency distribution showed that 70% of spat settled, in the frequency of 180-200 µm, in pH 8.1 (Fig. 5. 7). It was about 80 % in pH 8 and ambient pH 8.1. The size of larvae showed wide variation in pH 8.5.

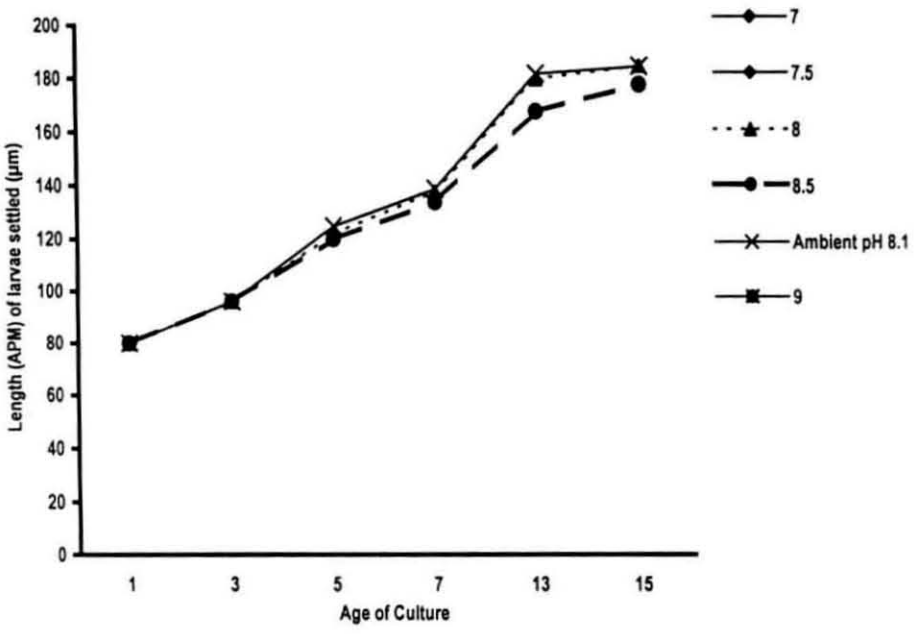
Table 5.6 Effects of different pH levels on clam larval growth and setting

pH → / No. of days ↓	Larval Growth and setting of Clam larvae				
	Mean size of larvae (µm)				
	7.0	7.5	8.0	8.5	8.1 (Ambient pH)
2	80 ± 0	80 ± 0	80 ± 0	80 ± 0	80 ± 0
3	80 ± 0	-	96 ± 1	96 ± 0	96 ± 0
5	-	-	122 ± 2	120 ± 2	125 ± 1
7	-	-	138 ± 2	134 ± 3	139 ± 2
11	-	-	180 ± 3	168 ± 2	182 ± 3
15	-	-	185 ± 2	178 ± 3	185 ± 2

Growth rate

The maximum growth rate (7.3 µm/day) of umbo was at pH 8.0 and 8.1 (Table 5. 7). In high pH, it was 5.0 µm/day. Thereafter, till settlement, a uniform range of 5.8, 5.7 and 5.5 µm/day respectively in 8.0, 8.1 and 8.5 pH levels were observed. The growth rate regression, which observed in the same period, is observed 0.845, 0.838 and 0.812 log µm/day. These values showed no significance among the treatment ($P \leq 0.05$).

Fig. 5. 6. Larval growth when reared at different pH levels till settlement



The observed overall growth rate on 15th day for pH 8.0, 8.1 and 8.5 was 7.0, 6.9 and 6.5 µm/day respectively. Growth rate was 0.863 log µm/day in case of 8.0 and 8.1 and 0.698 log µm/day in pH 8.5.

Fig. 5. 7. Size Frequency distribution of clam larvae reared and settled in different pH on day 15.

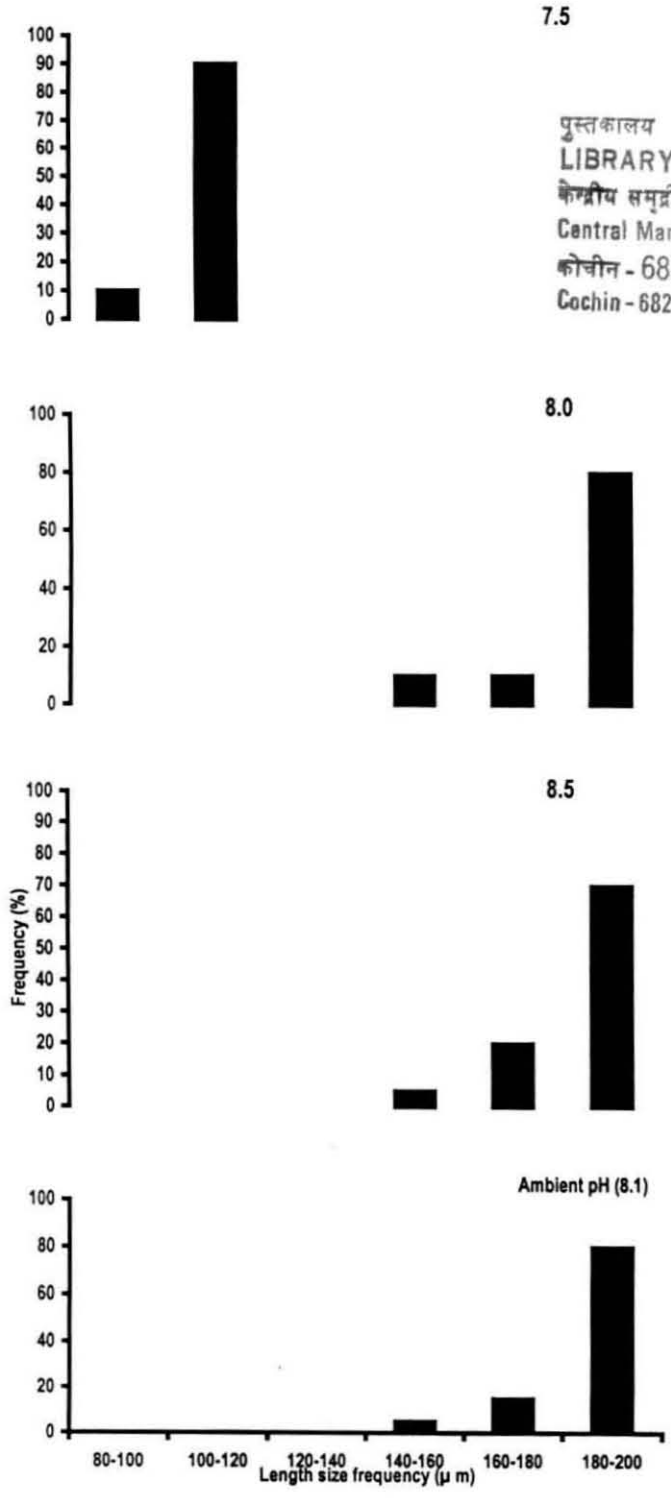


Table 5. 7. Growth rate of clam larvae during development in different pH

	Mean Growth rate (µm/day)			
pH	8.0	8.5	9.0	8.1 (ambient pH)
D – Umbo	7.3	5.0	-	7.3
Umbo-Spat	5.8	5.5	-	5.7
D - spat	7.0	6.5	-	6.9

Survival

The larvae reared at pH levels 8.0 and 8.1 shows maximum survival of umbo on day 6. The survival rate of umbo was 73.4 and 84.5 % in the above mentioned pH levels, while it was only 60.3 % in pH 8.5 (Fig. 5. 8). 90 % of umbo further developed and settled in the pH range of 8.0 and 8.1. However, a survival rate of 84 % was also observed in later stage of development in pH 8.5 than initial developmental stage (Fig. 5. 9).

The overall observed survival rate of spat that settled at pH 8.0, 8.1 and 8.5 was 66.8, 71.1 and 54.2 % respectively. No larvae in the pH 7.0, 7.5 and 9.0 were survived beyond *D* – stage.

No. of days taken for spat settling

There was not much variation on settlement days in various pH levels. It was observed that at control pH 8.1, the larvae showed an initial settlement on day 12 and the complete spat settled on day 14. In case of pH 8.0, settling started on day 13 and completed the process on day 15, where it was observed on 13th day and 15th day respectively in pH 8.5.

The inverse difference in the spat settlement in this experiment was due to low survival rate of spat.

Fig. 5. 8. Percentage of Survival of umbo during development and spat settled in different pH levels

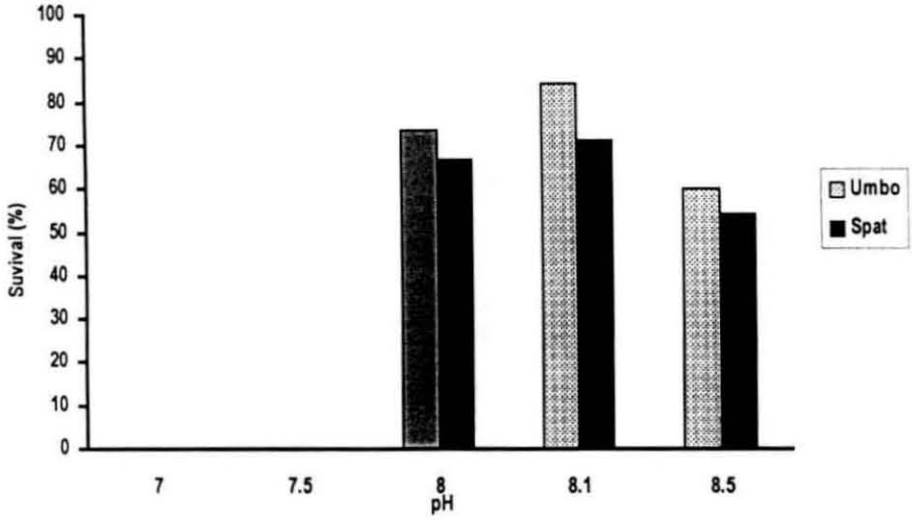
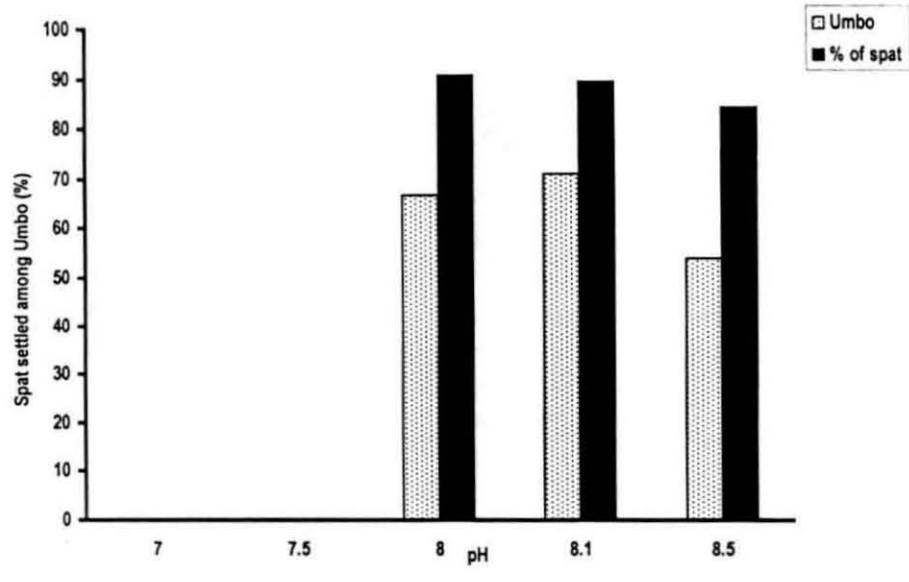


Fig. 5. 9. Percentage of Umbo settled during development in different pH levels



Algal cell consumption

There was no significant variation in algal cell consumption in 8.0 and 8.1 pH levels. The observed cell consumption in above mentioned pH levels were 85 and 88 % in *D* stage, 80 and 85 % in umbo and 85 and 88 % in spat respectively (Table 5. 8). The algal cells were intact in lower pH levels 7.0 and 7.5, but larvae did not survive in these pH levels. The same was also observed in pH 9.0. Table 5. 9 summarise the effect of pH during larval rearing of *P. malabarica*

Table 5. 8. Algal cell consumption during larval rearing at different levels of pH

Algal cell consumption (%)						
Stage	7.0	7.5	8.0	8.5	9.0	8.1 (Ambient pH)
D larvae	-	-	85	67	-	88
Umbo	-	-	50	75	-	85
Spat settled	-	-	85	75	-	85

Table 5. 9. Effects of different pH levels on clam larval growth and setting

	Larval Growth and setting of Clam larvae				
	Mean size of larvae (µm)				
pH → **No. of days ↓**	**7.0**	**7.5**	**8.0**	**8.5**	**8.1 (Ambient pH)**
2	**80**	**80**	**80**	**80**	**80**
3	**80**	**-**	**96**	**96**	**96**
5	**-**	**-**	**122**	**120**	**125**
7	**-**	**-**	**138**	**134**	**139**
11	**-**	**-**	**180**	**168**	**182**
15	**-**	**-**	**185**	**178**	**185**
Day of 1st setting	**-**	**-**	**14**	**13**	**13**
Final setting	**-**	**-**	**15**	**15**	**15**
Total No. of spat	**-**	**-**	**6675**	**5415**	**7113**
Spat production (%)	**-**	**-**	**66.8**	**54.2**	**71.1**

Spat / Juveniles

5. 3. 1. Effect of salinity on spat

Growth

The spat reared in different salinities showed significant increase in size with increase in salinity. Maximum shell length observed during the 30 days rearing was in the salinity 30 and 35 $^{o}/_{oo}$ with a mean size of 7.85 ± .65 and 7.70 ± 0.35 mm respectively. Lowest shell growth observed in salinity 10 and 20%. The observed mean shell length was 5.60 ± .30 mm and 5.70 ± 0.28 mm respectively. In high salinity 40 $^{o}/_{oo}$, spat survived were measured with a mean size of 5.9 ± 0.20 mm. The observed mean size of spat reared in different salnities is summarised in Table 5.10.

Growth rate

The highest growth rate was observed in salinity 30 $^{o}/_{oo}$ and lowest in 10 $^{o}/_{oo}$. The observed growth rate in salinity 10, 15, 20, 25, 30, 35, 40 and ambient 33 $^{o}/_{oo}$ were 0.09, 0.16, 0.19, 0.19, 0.23, 0.22, 0.16 and 0.22 mm/day respectively. The analysis of covariance showed not much significance between the treatments ($P \leq 0.05$).

Survival

The spat reared in all the salinities except 40 $^{o}/_{oo}$ survived. The percentage survival of spat reared in lower salinities 10 and 15 $^{o}/_{oo}$ was 95 % (Table 5. 10).

Table 5. 10. Mean size (APM in mm) of spat reared in different salinities (for 30 days)

No. of days ↓	Spat growth: (anterior posterior measurement) Mean (± s.d)							
	10 $^{o}/_{oo}$	15 $^{o}/_{oo}$	20 $^{o}/_{oo}$	25 $^{o}/_{oo}$	30 $^{o}/_{oo}$	35 $^{o}/_{oo}$	40 $^{o}/_{oo}$	33 $^{o}/_{oo}$
Initial size	0.900 ± .11	0.951 ± 0.12	0.98 ± 0.12	0.98 ± 0.10	1.02 ± 0.10	1.00 ± .03	1.02 ± .03	1.00 ± .04
Final size	3.54 ± .05	5.7 ± 0.05	6.63 ± .10	6.65 ± 0.11	7.85 ± .25	7.70 ± .18	5.9 ± .09	7.80 ± 0.22
% of survival	95	95	100	100	100	100	50	100

Algal cell consumption

The spat reared in salinity range 20-30 $^{o}/_{oo}$ showed high consumption of algal cells, while it was low in lower salinities 10 and 15 % initially. Later in these lower salinities also consumption of algal feed was same as spat reared in other salinity range. Maximum algal cell consumption was observed in salinity 30 and 33 %o with an average of 88.3 and 94 % respectively. The observed average consumption rate all together during the period is 58.3, 62.3, 65.3, 73.2, 88.3, 80.0, 61.0 and 94 % in salinity 10,15,20,25, 30, 35, 40 and 33 %o respectively (Table 5. 11).

Table 5. 11. Algal cell consumption during spat rearing at different salinities

Average Algal cell consumption (%)								
Salinity (%)	10	15	20	25	30	35	40	33 (Ambient salinity)
Day 1	35	35	58	75	85	80	50	91
Day 15	70	77	63	70	90	80	68	95
Day 30	70	75	75	75	85	80	65	95

5. 3. 4. Effect of pH on spat

Growth

The spat reared in pH 8.0, 8.5 and 8.1 (control pH) showed a good growth during the experiment. The mean anterior posterior measurement is observed was 6.70 ± 0.21, 6.75 ± 0.20 and 6.75 ± 0.25 mm respectively in above pH levels. None of the spat survived beyond day 4 in the pH 7.0, 7.5 and 9.0 in the present experiment.

Growth rate

Growth rate was slow (0.17 mm/day) in high pH 8.5. The observed growth rate was 0.19 mm/day in pH 8 and 8.1 (ambient pH). There was no significant variation statistically within the treatments ($P \leq 0.05$).

Survival

The spat reared in pH levels 8.0,8.5 and 8.1 showed 100% survival. The spat in low pH 7.0 and 7.5 did not grown beyond day 4. The same was also observed in high pH 9.0. This may be due to chemical reaction of Tris buffer, which added to raise the pH.

Algal cell consumption

The spat that survived in pH 8.0, 8.5 and 8.1 (ambient pH) consumed algal cells at an average rate of 90% of the feed, *N. salina* provided.

5. 4. Discussion

In natural environment, spawning and release of gametes is during favourable conditions, for maximum larval survival and continuity of species (Sastry, 1965). Environmental conditions affect larval growth and survival of many invertebrates (Kinne, 1963; 1964) including crustaceans (Mene *et. al.*, 1991; Brown and Jeffrey, 1992), echinoderms (Watts *et. al.*, 1982) and molluscs (Tettelbach and Rhodes, 1981; Zimmerman and Rechenik, 1991).

Larvae develop over a wide range of environmental conditions, where they inhabit. The duration of larval planktonic life typically ranges from about one to four weeks and is depended upon salinity, temperature, pH, available ration and other factors. This was proved in mussels, *Perna virids* (Bayne, 1976; Sprung, 1984). Numerous studies have been conducted on the effect of salinity on embryonic and larval development of temperate mussels, *Mytilus edulis* (Hris-Brenko and Calabrese, 1969) and *Perna viridis* (Shau-Hwai Tan, 1997).

Most works were done in temperate species. It has been found that temperate species have seasonal restriction period, while it is prolonged one in tropical waters (Geise, 1969). In tropical conditions like Indian waters, spawning in bivalves is influenced by the changes in salinity over the animal beds (Rao, 1956; Desmukh, 1972). Naghabhushanam and Mane (1975) reported that increase in salinity initiated spawning in *Crassostrea madrasensis* from the east coast, and *Meretrix meretrix* and *Katelysia opima* from the south west coast respectively.

Studies on the reproductive cycles of clams from Indian coastal waters have been carried out on *Meretrix casta* (Hornell, 1922 and Abraham, 1953), *Donax faba* (Alagarswami, 1966), *Donax cuneatus* (Rao, 1968), *Katylesia opima* (Naghabhushnam and Mann, 1975), *Paphia laterisulca* (Nagabhushanam and Dhamne, 1977) and *Paphia malabarica* (Rammohan and Velayudhan, 1998).

Clams being found mostly in the estuarine environment are naturally subjected to fluctuating environment conditions, which exert great influence on their life stages. The clam *Paphia malabarica*, the species in the present study is found in estuary and backwaters of the coastal belt. There are great fluctuations

during tide, especially during low tide, where the clam beds get exposed to air. During monsoon, the salinity of the water over these beds may remain low for a long period. So clams in such area have adaptation to overcome these changes. Survival and behavior of clams in low salinities have been studied in temperate conditions. Little work is done in tropical conditions, especially in larval stages.

It has been reported that salinity influences the growth and breeding of clams, *Meretrix meretrix* and *Katelysia opima* (Ranade and Kulkarni, 1964). Effect of salinity on larvae of other bivalves was reported in tropical waters. Shau Hwai Tan (1997) reported that larvae of *Perna viridis* could thrive in 16-30 $^{o}/_{oo}$. In this salinity range, fertilized egg developed to '*D*' larval stage with a maximum survival of 84-91 %. Similarly, responses were reported for the species *Mytilus viridis* (Lim, 1992) within a range of 24-30 %; *M. viridis* with a range of 30 $^{o}/_{oo}$ (Tham *et. al.*, 1972) and in *Mytilus edulis* (Bayne, 1965). It was reported a salinity range of 28-35 $^{o}/_{oo}$ is good for normal development of larvae of *Mytilus californianus* (Young, 1941). However, higher salinity 40 $^{o}/_{oo}$ was reported as the optimal salinity for development of eggs in *Mytilus galloprovincialis* (Hris Brenko, 1974).

In the present study, the larvae were reared and developed in lower salinities too, but the survival rate is low. Larval survival was low in lower salinities i.e., 10- 25 $^{o}/_{oo}$, where as it is high in 25 –30 $^{o}/_{oo}$. The larvae of *Paphia malabarica* reared in the ambient salinity 33 $^{o}/_{oo}$ also showed high survival of 85 %. Walne (1965) observed lower growth of *Mytilus edulis* larvae at 15 $^{o}/_{oo}$, slow growth at 24 % and a higher growth at 30-32 $^{o}/_{oo}$. Anuradhakrishnan (1993) reported a survival rate of 70 % in larvae of *Pinctada fucata* in salinity 28-30 $^{o}/_{oo}$.

Also reported that in low salinities, survival was low and development was slow in higher salinities. ie., 35-40 $^{o}/_{oo}$. Albentosa *et al.* (1999) reported a high survival in clam *Venerupis pullsatra* reared in salinity 27 $^{o}/_{oo}$.

The salinity also affects the settling of larvae. Early settlement in *P. viridis* was observed in salinity 30 $^{o}/_{oo}$ on day 20, while it was delayed for another 3-5 days in lower salinities. In the present study, the larvae reared at ambient salinity 33 $^{o}/_{oo}$ and 30 $^{o}/_{oo}$ shows early initial spat settlement on day 9 and that, complete settlement on day 11. In lower salinities, the settlement was prolonged for another 2- 3 days, with low survival.

In case of algal consumption, it was reported that in lower salinities, consumption rate was high (20-25 $^{o}/_{oo}$) in larvae of pearl oyster, *Pinctada fucata* (Anuradhakrishnan, 1987). The consumption of algal cells in early stages was about 60 % and decreased with development. But, it is reported that the phytoplankton can withstand wide fluctuations of salinities (Walne, 1965). In the present study, the algae, *Nannochloropsis salina* was used. The mass culture of this species was carried out in the salinity range 28-30 $^{o}/_{oo}$. It was observed that 90 % algal cell consumed in salinity 30 $^{o}/_{oo}$ and only a marginal reduction in low salinities. Moreover, the size of the particle forms a factor. This algae being 2- 3 µm, was easily filtered and consumed by larvae for their development.

With regard to pH, spat settled at pH 8.0 and ambient pH 8.1 shows a maximum mean size of 185 µm. High pH of 8.5 also showed a mean size of 178 µm, but the survival was only 54.2 %. The initial spat settlement was observed on day 12 in pH 8.0 and the complete spat settled by day 14.

Davis and Calbrese (1964) reported the normal development of adult clams *Mercenaria mercenaria* and *Crassostrea virginica* at pH 7.0 –7.5. The range of normal growth was narrower than survival. Rapid growth was reported in pH 8.25 - 8.50.

Davis *et al.* (1966) indicated the possibilities of algal death due to pH variations. Jaime (1983) reported phytoflagellates grew best at pH 8.0 while acidic pH was unstable. Most of the marine species grew at pH range between 6.5 - 8.0. The algal species used in the present study were cultured in seawater with a pH range of 7.9 - 8.2. Algal consumption of larvae in this pH range was showed normal development and settlement. The spat reared in these levels of pH also showed the same result.

In the present study, from the results of the experiments conducted, it could be concluded that the larvae of the yellow clam *Paphia malabarica* can be reared in salinity range 25-33 $^{o}/_{oo}$ and pH 8.0 - 8.5.

Summary

Summary

To standardize the hatchery feed protocol for larval rearing of *Paphia malbarica*, the present study emphasized on optimum algal feed requirement of larvae at all stages of development. As the algal feed fulfills, the physiological and biological needs during development, the suitable micro algal production in a hatchery system could be benefited for the larval rearing and spat production on a commercial basis.

In most bivalve hatcheries, the phytoflagellate *Isochrysis galbana* was used as feed in early larval stages, without considering the optimum requirement of this species. The present study revealed that a cell concentration of 5 x 10^3 cells/ml is the ideal density for larval rearing of *P. malabarica*.

The present work, with an objective, to evaluate the efficiency of other micro algae such as *Nannochloropsis salina, Tetraselmis gracilis, Dicrateria inornata, Chaetoceros calcitrans* and *Dunaliella salina* as an alternative feed to *I. Galbana*. Among these micro algae, it is established that *Nannochloropsis salina* could be used for larval rearing and spat production, since it showed good result in terms of early settlement (day 9 –11), survival (83 %) and high growth rate (15 μm/day) when fed as single diet.

Among the combinations of two algal species tested, *N. salina* with *I. galbana* yield best result in terms of survival and growth in different stages of larval development. In order of merit, it is suggested that a combination of *N. salina* with *Tetraselmis gracilis, Dicrateria inornata* or *Chaetoceros calcitrans* proved that it could be used as ideal combination of algal feed.

As the cell size plays a critical role in filtration and clearance rate, the low cell size (2 μm), micro algae, *Nannochloropsis salina* shows high consumption among all the species tested. Base on the cell size, preference of larvae on the algae tested is in the order *Nannochloropsis salina* > *Isochrysis galbana* > *Dicrateria inornata* > *Chaetoceros calcitrans* > *Dunaliella salina* > *Tetraselmis gracilis.*

With regard to biochemical composition, the protein remains high among proximate principles of micro algae. The dietary protein thus fed to clam larvae, also reflected in their composition during larval development. The protein was high in each larval stage i.e., *D* larva, umbo and settled spat, than carbohydrate and lipid. The combination of high proteinacious (51 %) micro alga *Nannochloropsis salina* with *Isochrysis galbana* shows high protein value in each stage of development. The other algal combinations, *N. salina* + *Dicrateria inornata, N. salina* + *T. gracilis, I. galbana* + *Dicrateria inornata, I. galbana*+ *T. gracilis* and *Dunaliell salina* + *N. salina* also proved to be good combination for the larval rearing and spat production of *P.*

malabarica. From the results obtained in the present study, it is suggested that *N. salina* could be used as an alternative to *I. galbana*.

The environmental factors, salinity and pH, also forms an important aspect for the larval rearing and spat production. The salinity range 30 – 33 $^{o}/_{oo}$ and pH 8.0 – 8.5 could be ideal range for the larval rearing and spat production and also seed culture of *Paphia malabarica*.

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Appendix

List of Publications

R. Gireesh, K.S. Smitha, K.B. Bindhu and C.P. Gopinathan. 2001. *Dunaliella salina* – an unconventional live feed. In: *Perspective in Mariculture*. JMBAI, pp **235-240**.

P. Kaladharan, **R. Gireesh** and K. S. Smitha. 2002. Cost effective medium for the laboratory culture of live feed micro algae. *Seaweed Research and Utilization*. **24 (1):** 35-40.

Dunaliella salina - an unconventional live feed

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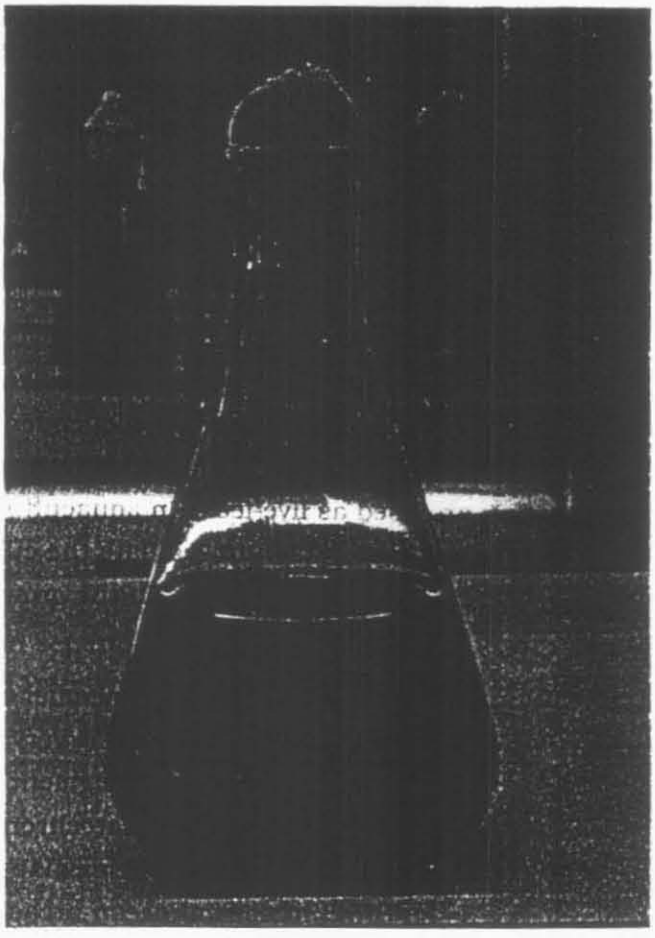
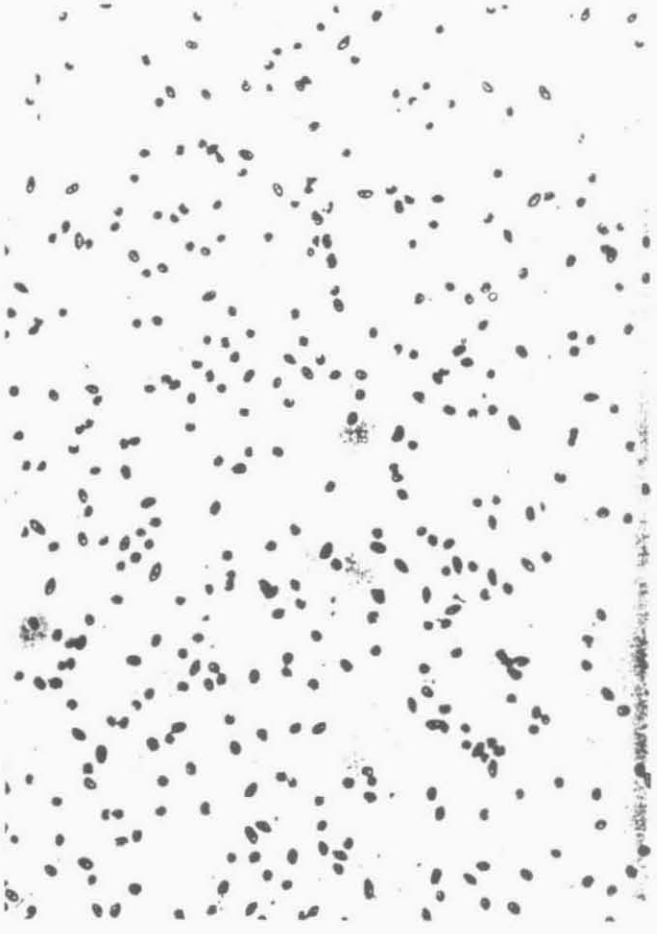


ABSTRACT

Live feeds play an important role in aquaculture operation. Presently, the groups of live feeds employed in the culture systems are limited to a few species of phyto and zooplankters. Dunaliella salina, *(greenmicroalga), a member of Chlorophyceae, is an unconventional live feed. The culture of this species is presently limited to laboratory experimental stage only.*

In view of the paucity of studies on the culture and utilization of Dunaliella salina, *the present investigation was undertaken to explore and estimate the potential use of this species. The results of rearing of juvenile clams with* Dunaliella *as live feeds are presented and discussed.*



Introduction

In aquaculture systems, several live feeds are used presently such as *Chaetoceros, Isochrysis, Skeletonema*, etc. The hatchery rearing of prawn/ mollusc larvae is dependent on these live feeds. There are innumerable species of phytoplankton in our waters; but only very few of them are used as live feeds in aquaculture. In view of this, the present study was undertaken to evaluate the culture possibilities of *Dunaliella salina* and to study its effect on the growth, survival rate and performance in juvenile clams.

Dunaliella salina is a member of Chlorophyceae; a green halotolerant (ie., thrives in media with a very board range of salt concentrations) microalga. The dominant pigment - chlorophyll, is masked by the presence of a pigment - haematochrome. It accumulates large amounts of commercially valuable chemicals - glycerol b and - carotene. *Dunaliella* is cultured in coastal ocean areas in large outdoor ponds in regions of high solar radiation and moderate temperatures. An attempt has been made in this brief work to evaluate the performance of *Dunaliella salina* in laboratory / hatchery conditions.

Materials and methods

Four live feeds *viz, Dunaliella, Chaetoceros, Tetraselmis* and *Nanochloropsis* were cultured in laboratory conditions using Walne's medium in sterilized seawater at 30 ± 2ppt salinity.

Juvenile clam of *Villorita cyprinoides* (7.52g average initial weight) were brought from a local farm off Cochin and reared in 8-10 ppt salinity under labouratory conditions. The clams were acclimatized for rearing in 8-10 ppt salinity under laboratory conditions. The clams were acclimatized to the rearing conditions for over a period of one week. Prior to starting of the experiment, the clams were starved for 48 hrs. The clams were then grouped into four goups; each group containing 10 animals (Table 1). Each group was fed on separate feeds to study the comparative efficiency of *Dunaliella* with respect to the other conventional live feeds.

Table 1. Groups, feeds and feeding rate used in the experiment

Groups	Feeds	Feeding rate
Group I	*Dunaliella*	500-700 cells/ml
Group II	*Chaetoceros*	-do-
Group III	*Tetraselmis*	-do-
Group IV	*Nanochloeopsis*	-do-

The animals were maintanined in separate tubs with 3 l of filtered seawater. The feed ration was divided into two and given at intervals of 8hrs. 100% water exchange was done every day. The feeding rate (ingestion rate) was determinded as :

$$\text{Feeding rate or ingestion rate} = \frac{C1 - C2}{nt} \times V \times 60$$

where, C1 - initial cell concentration (cells/ml)

C2 - final cell concentration (cells/ml)

V _ Volume of water (l)

t _ duration of the experiment

n _ no. of animals.

The animals were reared with the respective feeds for a period of 15 days. At the end of the experiment, the animals were sacrificed and their biochemical composition estimated. Growth and survival rate was also studied. Biochemical assays were carried out to estimate the protein, carbohydrate and lipid contents.

- Protein was estimated following the procedure of Lowry *et al.* (1951). The values were obtained by comparing with standard graph.
- Carbohydrate was estimated using Glucose as standard.
- Fat (lipid) was determined using gravimetric method.

Simultaneously a group of 10 clams were maintained as control for the same length of period as that of the experimental only. All conditions were the same for this group also, except that they were not fed any feed.

Culture of *Dunaliella* : The candidate species, *Dunaliella salina*, was cultured in three different media for a period of 15 days at 30 ppt salinity and 24°C.

Medium I - Walne's medium

Medium II - Miquel's medium

Medium III - Enriched seawater medium (modified 'F' medium).

20 ml of *Dunaliella salina* was inoculated into 500 ml of filtered and sterilised seawater containing the respective medium. The culture was carried out in triplicates. (1 ml of inoculum contains approximately 500 cells/ml).

Ressults

Feeding experiment: The performance of each group of clams was monitored. The efficiency of the feed was determined in terms of growth, survival and biochemical changes of the clams. A summary of the results obtained is shown in Table 2. No mortality was reported with any of the feeds. However, the results were discouraging with respect to *Dunaliella* fed clams. Clams fed on *Dunaliella* showed comparatively less protein and carbohydrate contents. However, they showed better lipid profiles, as explained elsewhere. The growth rate was more with *Dunaliella* fed clams as seen in Table 2; but biochemical estimation gives comparatively lower values for *Dunaliella* fed clams.

Table 2. Growth and biochemical composition of the differet groups of clams

Group	Feed fed	Av. initial weight(g)	Av. final weight(g)	% growth	Protein (/g)	Carbohydrate(/g)	Lipid*
I	*Dunaliella*	8.69	8.86	1.96	21µg	0.55µg	0.249
II	*Chaetoceros*	11.42	11.45	0.263	30µg	1 µg	0.249
III	*Tetraselmis*	9.73	9.79	0.617	28µg	0.8µg	0.109
IV	*Nanochlor*	12.28	12.30	0.163	10µg	0.4µg	0.135

* Expressed as mg lipid/ gm tissue.

Culture of *Dunaliella* : The performance of each media was determined taking into consideration the following facts:

- incubation period, ie, the time taken for the culture to start growth. - exponential period - the duration for which the bloom lasts.
- final maximum no. of cells/ml at the time of harvest.

Accordingly, the medium with the least incubation period and highest exponential period along with better biochemical results is recommended as the ideal one. The results of culturing *Dunaliella* with the different media is given in Table 3.

Table 3. Results of *Dunaliella* culture with different media

Media	Initial no. of cells/ml	Final no. of cells/ml	Incubation period (days)	Exponential period (days)	Biochem. compostion		
					Protein µg/g	Carbo-hydrate. µg/g	Lipid mg/g
Walne's	500	13x103	3 to 4	one month	17.5	14	0.773
Miquel's	-do-	*	two weeks	one week	-	-	-
Modified F	-do-	*	-	-	-	-	-

* not determined as culture did not develop.

In the case of Walne medium blooming started within three to four days after inoculation. With Miquel's medium, blooming took ten to eleven days and declined in about two days time. With modified 'F' medium, there was no blooming at all. Since, the amount of sample that could be obtained from these two media ('F' and Miquel media) were very less, no biochemical estimation of these could be carried out. However, biochemical studies were carried out with *Dunaliella* cultured in Walne medium.

Discussion

Clams fed with *Danaliella* showed a higher percentage of growth (1.96%) compared to the other groups. Also, *Dunaliella* fed clams showed an increased lipid content (0.2488 mg/g) compared to other groups. This is attributed to the high levels of glycerol accumulated by *Dunaliella* in culture conditions (Della et al. 1995). Studies on feeding *Artemia* with *Dunaliella* have given large scale mortality. Similarly, with shrimps also mortality and collapse of culture has been recorded (C.P. Gopinathan, unpublished).

The results indicate that *Dunaliella* is not a substitute for the al-

ready widely used live feeds such as *Chaetoceros* and *Tetraselmis*. But, it can be incorporated as a supplementary live feed in molluscan culture. The study also indicates that *Dunaliella* is not toxic to juvenile and adult calms. The effect of rearing larval molluscs with *Dunaliella* remains to be studied. As regards the lower biochemical values obtained with *Dunaliella*, it can be mentioned that *Dunaliella* uses a lot of its energy on photoconversion - where the dominant green pigmet is converted into Xanthophyll. Also the higher lipid values explains the lower protein and carbohydrate values.

The results indicate that Walne medium is the ideal one for culturing *Dunaliella*. With this medium a good bloom of the culture and excellent exponential growth is obtained.

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Cost effective medium for the laboratory culture of live feed micro algae

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ABSTRACT

Extracts of the green seaweed *Ulva lactuca* promoted the growth and the mutiplication of three species of micro algae, *Tetraselmis gracilis, Isochrysis galbana* and *Chaetoceros calcitrans* 250-325% more than those cultures supplemented with vitamins (B_1 and B_{12}). When these microalgae were cultured in seawater supplemented with varying levels of extracts of garden soil and *Ulva lactuca*, *Isochrysis galbana* and *Tetraselmis gracilis* registered 16% and 58% increase in growth respectively and 19% decrease in growth by *Chaetoceros*. The results are discussed in the light of preparations of a low cost effective and ready to use recipe for the mass culture of these live feed organisms.

Introduction

With the rapid increase in aquaculture production, there is an ever-growing interest in live feed culture. Live feeds constitute the inevitable input in hatchery operation of any aquaculture system. Being the primary link in the food chain, phytoplankton (micro algae) among live feeds plays a very important role. Thus the culture and maintenance of these feed organisms becomes equally important. Gopinathan(1982) has described the batch culture method for the mass culture of phytoplankton for shellfish hatcheries. Although batch culture is relatively easy to carry out, its efficiency is very poor and the cultures are prone to crash. Considering the advantages of continuous and semi continuous culture systems over the traditional batch culture systems, a number of workers (Persoone and Sorgelos, 1975; Boussiba *et. al.*, 1988; Janes and Al Khars, 1990;Feberga *et. al.*, 1996;Lambade and Mohamed, 2001) have reported on several designs for the continuous production of micro algae in high densities.

Like culture methods, culture media also play a major decisive role in live feed culture. There are a number of conventional media, such as, Walne's, Scheiber's, Miquel's etc., being used for the culture and maintenance of micro algae in the laboratory as well as in hatchery. These media contain inorganic recipes and procurement of the ingredient chemicals is tedious and often expensive. It is imperative that to make the hatchery production of shellfish and finfish profitable, the essential operational inputs are

to be minimized for all stages of hatchery programmes including feed development by adopting low cost productions. Although Kumaraswamy Achari and Kaumudi Menon (1993) have reported a simple medium to isolate and culture species of *Chromulina, Pavlova* and *Chlorella*, the new medium contains higher concentrations of phosphate and vitamins. An attempt has been made in the present study to formulate a new medium for the culture of micro algae, which is derived from organic ingredients, which are cost effective and are easily available. The study aims at the formulation of a low cost culture medium, which can be easily prepared and at the same time can offer the desired growth rate.

Materials and Methods

The medium comprised of extracts of the green seaweed *Ulva lactuca* and garden soil.

Preparation of *Ulva* extract

100g wet weight of *Ulva lactuca* was cut into small pieces and boiled in about 500 ml distilled water for about 20 minutes with constant stirring. It was then squeezed and filtered through a 5μ mesh. The final extract thus obtained was made up to 500 ml in a standard flask. This was then autoclaved, cooled and refrigerated for further use.

Preparation of garden soil extract

One kg of garden soil was sieved to remove stones and such other materials. This was then boiled in one litre distilled water for about 30 minutes to get the extract. It was filtered, autoclaved, cooled and then kept under refrigeration for further use.

Experimental set up

Two experiments were conducted with three species of micro algae namely *Tetraselmis gracilis, Isochrysis galbana* and *Chaetoceros calcitrans*, which are the most commonly used live feeds.

Experiment I consisted of culturing these species of micro algae in Walne's medium (Walne, 1974) with *Ulva* extract. The extract was added at levels of 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 to the culture flasks containing the inocula and Walne's medium (devoid of vitamins).

Experiment II consisted of growing these species with *Ulva* extract and gardensoil extract (0.5-5.0ml). In both the experiments, Walne's medium enriched with vitamins served as the control. The particulars of experimental set up was given in Table 1.

Both the experiments were carried out for 8 days duration. Counts/ml were recorded on zero days and then on every alternate day to determine the growth and multiplication of the culture, using a haemocytometer with improved Neubaeur ruling. Measurement of growth, rate of multiplication/day was calculated as per Herraro *et. al.* (1991). The growth and net yield were analysed using ANOVA for test of significance (Snedecore and Cochran, 1967) and the analysis was done using SPSS/PC software.

Results:

The Experiment I showed that 1-2 ml of *Ulva lactuca* extract supplemented to Walne's medium increased the net growth of 315% in *Isochrysis galbana* ($P<0.05$), 323% in

Table 1. *Details of experimental setup*

	Inoculum	Seawater	*Ulva* extract	Soil extract	Walne's medium
Experiment I	10ml	85-90ml	0.5-5.0ml	Nil	2ml
Experiment II	10ml	80-90ml	2.0ml	0.5-5.0%	Nil
Control	10ml	90ml	Nil	Nil	0.2ml

Table 2. *Effect of extract of* Ulva lactuca *on the growth of* Isochrysis galbana *(n x 10^4) cultured in Walne's medium*

Treatment (ml)	Days One	Three	Five	Seven	Net growth (%)
0.5	28.50	51.70	92.60	66.75	234
1.0	27.50	62.50	80.00	96.20	350
2.0	16.75	19.25	57.50	128.50	767
3.0	19.50	21.50	86.50	88.00	451
4.0	18.33	n.d	83.50	88.70	484
5.0	17.60	n.d	76.00	82.00	470
Control	18.70	21.00	88.50	84.50	452

n. d. - not detected

Table 3. *Effect of extract of* Ulva lactuca *on the growth of* Chaetoceros calcitrans *(n x 10^4) cultured in Walne's medium*

Treatment (ml)	Days One	Three	Five	Seven	Net growth (%)
0.5	33	23.75	42.25	103.0	312
1.0	30	64.75	136.5	166.5	555
2.0	30	26.50	60.25	73.30	245
3.0	30	17.25	65.75	59.75	171
4.0	32	87.75	48.25	61.0	190
5.0	31	43.75	51.75	53.75	173
Control	25	34.25	47.75	58.0	232

Table 4. *Effect of extract of* Ulva lactuca *on the growth of* Tetraselmis gracilis *(n x 10^4) cultured in Walne's medium*

Treatment (ml)	Days One	Three	Five	Seven	Net growth (%)
0.5	16.0	27.0	36.0	42.0	263
1.0	19.0	29.0	34.0	84.0	442
2.0	11.0	18.0	25.0	66.0	600
3.0	11.0	23.0	22.0	45.0	409
4.0	16.0	n.d	27.0	40.0	250
5.0	15.0	22.0	31.0	40.0	267
Control	12.0	22.0	32.0	41.0	342

n. d. - not detected

Table 5. *Effect of extract of* Ulva lactuca *(2%) and garden soil (0.5 - 5.0 %) on the growth of* Isochrysis galbana *(n x 10^4) Cultured in Walne's medium*

Treatment (ml)	Days One	Three	Five	Seven	Net growth (%)
0.5	13.25	24.5	44.5	45.75	345
1.0	11.75	34.00	42.25	53.00	451
2.0	14.75	29.70	21.00	63.00	427
3.0	15.50	26.00	39.00	51.03	329
4.0	15.82	35.25	45.50	57.00	360
5.0	19.50	41.75	38.25	58.50	300
Control	19.25	23.70	88.50	83.75	435

Table 6. *Effect of extract of* Ulva lactuca *(2%) and garden soil (0.5 - 5.0%) on the growth of* Chaetoceros calcitrans *(n x 10^4) Cultured in Walne's medium*

Treatment (ml)	Days One	Three	Five	Seven	Net growth (%)
0.5	19.00	32.00	32.75	39.25	207
1.0	15.25	26.25	33.00	40.00	262
2.0	16.25	20.25	28.75	35.00	215
3.0	18.00	31.25	25.50	38.25	213
4.0	19.50	22.50	37.75	48.75	247
5.0	17.00	37.00	35.00	35.50	206
Control	20.00	33.50	45.75	56.25	281

Table 7. *Effect of extract of* Ulva lactuca *(2%) and garden soil (0.5 - 5.0 %) on the growth of* Tetraselmis gracilis *(n x 10^4) Cultured in Walne's medium*

Treatment (ml)	Days One	Three	Five	Seven	Net growth (%)
0.5	15.00	25.70	23.83	36.00	240
1.0	10.00	17.14	27.00	31.00	310
2.0	12.00	25.00	32.00	33.00	358
3.0	14.00	37.43	44.00	42.00	300
4.0	18.00	40.33	45.00	46.00	256
5.0	17.00	41.00	46.00	45.00	264
Control	14.00	24.00	33.00	42.00	300

Chaetoceros calcitrans (P< 0.05) and 258% in *Tetraselmis gracilis* (P<0.10) higher than their respective controls (Tables 2, 3 and 4; Fig. 1). Levels higher than 2ml extract of *Ulva lactuca* did not enhance the growth and multiplication of micro algal cells proportionately.

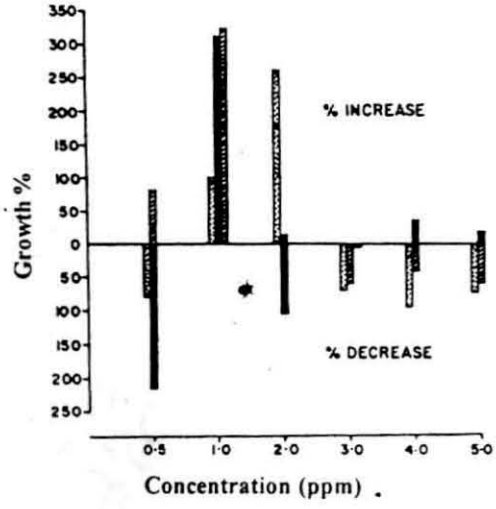

Fig. 1. *Effect of extract of* Ulva lactuca *on the growth of live feed algae cultured on Walne's medium*

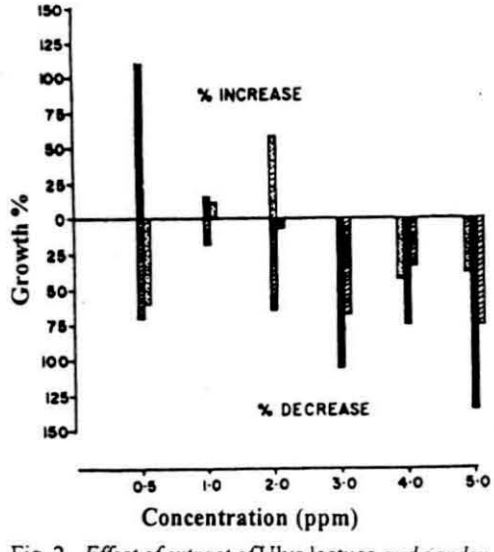

Fig. 2. *Effect of extract of* Ulva lactuca *and garden soil on the growth rate of live feed algae cultured in sterile seawater*

Culture of these micro algal cells carried out in the Experiment II in the seawater supplemented with extracts of gardensoil and *Ulva lactuca*, without any inorganic salts and vitamins resulted in considerable increase (Tables 5-7; Fig. 2) in cell numbers than their controls (Walne's medium). However, the net increase in cell numbers on the 7th day in 2% *Ulva lactuca* and 4 % soil extracts showed 16 % increase in *Isochrysis galbana* and a combination of 2 % *Ulva* extract and 4 % soil extract showed a net increase of 58 % in *Tetraselmis gracilis*(P<0.05), whereas cultures of *Chaetoceros calcitrans* showed 19% decrease (P<0.05) than the control (Table 5-7; Fig.2).

Discussion

Ulva lactuca is one of the most commonly available green seaweed along the seacoast and hence there is practically no difficulty in collecting them, as they inhabit the intertidal regions of the coastal areas. Chemical composition of *Ulva lactuca* is known from Chennubhotla *et. al.* (1991) and that of garden soil extract from Thompson and Troch (1979). Experiments on growth efficiency may offer some valuable clues in regard to the ecological success of an organism (Kinne, 1960). The present study indicates that extracts of the seaweed and the soil are rich in nutrients, which are cost effective and easy to obtain.

Addition of extracts of *Ulva lactuca* to Walne's medium (without vitamins) for the culture of *Isochrysis, Chaetoceros* and *Tetraselmis* enhanced the growth and multiplication of cells considerably (Tables 2-4). However, soil extract in the place of inorganic salts did support the growth, more or less comparable to the control although, not proved superior to the control (Tables 5-7) indicating the possibilities of culturing micro algae in seawater with these extracts only. It is observed that higher concentrations of *Ulva* extracts beyond 2 % and 4 % of soil extract are not favouring the growth and multiplication of microalgal cells but enables to achieve their exponential phase quicker than the other treatments (Tables 5-7)

The sterile extracts of *U. lactuca* and garden soil can be stored for about six months in the form of a ready to use ampoule mixed in appropriate proportions or as separately, that can form a medium when heated with known quantity of filtered seawater. This simple medium stands in sharp contrast to the widely used commercial media in terms of economic viability as the cost of production for this new medium would be just 1/10th required for the preparation of the commercial medium.

The performance of this simple medium can perhaps be improved by mixing the extracts of many other seaweeds belonging to Rhodophyceae and Phaeophyceae, also at various proportions. The major advantage of this new medium for culturing marine micro algae (live feeds), besides cost effectiveness, is that this medium reduces the risk of accumulation of inorganic salts in the micro algal cells and thereby their transport into the feeding organisms as well as into the culture environment.

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